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14. ABSTRACT
Prostate cancer is the second leading cause of cancer death in the United States. Although radiotherapy (RT) is one of the two curative treatments for prostate cancer patients, approximately 10% of low-risk cancer patients and 30-60% of high-risk prostate cancer patients experience biochemical recurrence within five years, among them 20% die in 10 years. The proposed research is based on the hypothesis that targeting protein arginine methyltransferase 5 (PRMT5) can sensitize primary and recurrent prostate cancer cells to RT. During the third grant period, we generated stable cell liens to induc bly express PRMT5 shRNA using DU-145 cells. These stable cell lines will be used to conduct proposed in vivo experiments. As we previously found that inh bition of PRMT5 by a specific inhibitor BLL3.3 did not sensitize radiation-resistant sublines to several chemotherapeutic agents, we tested whether inhibition of PRMT5 can sensitize these cells to ionizing radiation. While BLL3.3 did sensitize parental LNCaP and DU-145 cells to ionizing radiation, inh bition of PRMT5 did not sensitize radiation-resistant cells to radiation. These results collectively suggest that PRMT5 may not be involved in radioresistance in these radiation-resistant cells. Therefore, future search for additional mechanisms is warranted. Continuing previous analysis of PRMT5 expression in prostate cancer tissues, we have also found that PRMT5 expression correlates with AR expression at both mRNA and protein levels. This is very significant given our novel finding that PRMT5 epigenetically regulates AR transcription. In support of this, we have gained additional mechanistic insight that the transcription factor SP1 may recruit PRMT5 to the AR promoter through ChIP studies. As these results point to the importance of PRMT5 expression level in prostate cancer tissues, we have continued to characterize transcriptional regulation of PRMT5 by NF-Y, and we have demonstrated that the PKC/c-Fos signaling is a negative regulator of the NF-Y/PRMT5 axis in prostate cancer cells. Consistent with these cell-based studies, the expression level of several PKC isozymes inversely correlates with PRMT5 expression at the mRNA level in prostate cancer tissues. This represents the first study to link cell signaling to the regulation of PRMT5 expression.

15. SUBJECT TERMS
Prostate cancer, LNCaP, DU-145, ionizing radiation, PRMT5, PC-3, CREB, NF-Y

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Table of Contents

Page

1. Introduction…… 4
2. Keywords…… 5
3. Overall Project Summary…….... 6
4. Key Research Accomplishments…… 12
5. Conclusion…… 13
6. Publications, Abstracts, and Presentations…….…….. 14
7. Inventions, Patents and Licenses…… 14
8. Reportable Outcomes..…… 14
9. Other Achievements…… 14
10. References…….. 15
11. Appendices……. 17

1. Introduction

Prostate cancer remains the number one cancer diagnosed in men (except skin cancer), and it is estimated that there will be 220,800 new cases diagnosed and 27,540 deaths in the US in 2015 according to the American Cancer Society report. Radiotherapy (RT) is an important primary treatment for old patients with low-risk prostate cancer, the standard primary treatment for high-risk prostate cancer when combined with androgen deprivation therapy (ADT), and the major salvage therapy for local recurrence after surgery [1-5]. In addition, surgery plus adjuvant RT also demonstrates survival benefits when compared with surgery alone [1, 6, 7]. Despite that the majority of patients can be cured by RT, approximately 10% of patients with low-risk cancer and up to 30-60% of patients with high-risk cancer experienced biochemical recurrence within five years after RT, and among them 20% of patients died in 10 years [8-11]. Similar rate of recurrence was observed after surgery [12, 13]. Given that 96% of prostate cancer patients are present as localized disease in the US [14] and that most recurrent tumors are local recurrence [15], failure in controlling these localized primary and recurrent prostate cancers eventually leads to disease progression and contributes to the majority of prostate cancer deaths. Thus, developing effective primary and salvage RT for prostate cancer patients will have a huge impact on reducing prostate cancer mortality.

Protein arginine methyltransferases (PRMTs) are a family of proteins involved in post-translational modifications of histones and non-histone proteins [16, 17], mRNA splicing, nuclear-cytoplasmic shuttling, DNA damage response, and signal transduction [18]. Recent studies have further demonstrated that PRMT5 is involved in the DNA damage response by epigenetically modulating target gene expression or by regulating the function of proteins that are involved in the DNA damage response [19-21]. However, it remains uninvestigated how PRMT5 is involved in prostate cancer development, progression, and therapeutic responses. Based on the findings in the literature and the preliminary studies, it is hypothesized that radiation-induced or pre-existing PRMT5 overexpression contributes to the resistance of prostate cancer cells to RT in both primary and recurrent prostate cancer. The objective of the proposed research is to determine whether targeting PRMT5 can sensitize primary prostate cancer to RT, and can reprogram therapy-resistant recurrent prostate cancer to therapy-sensitive prostate cancer. Three specific aims are proposed in this project. **Aim 1** will determine that targeting PRMT5 can sensitize prostate cancer cells and prostate cancer xenograft tumors to fractionated ionizing radiation (IR) *in vitro* and in nude mice; **Aim 2** will determine that targeting PRMT5 can sensitize radiation-resistant prostate cancer cell sublines and recurrent xenograft tumors to radiation and chemotherapy *in vitro* and in nude mice; and **Aim 3** is to establish the clinical correlation between the expression level of PRMT5 and radioresistance and tumor recurrence in human prostate cancer patients. Under the support of this award, we have made the following progress during the third grant period (Aug 1, 2014 – July 30, 2015).

2. Keywords

Prostate cancer, LNCaP, DU-145, PC-3, PRMT5, CREB, ionizing radiation, NF-Y

3. Overall Project Summary

Task 1. Aim 1: To determine that targeting PRMT5 can sensitize prostate cancer cells and prostate cancer xenograft tumors to radiation *in vitro* and in nude mice (Months 1-18)

1a. Generate lentivirus for making doxycycline-inducible cell lines using LNCP, DU-145 and PC-3 cells (Months 1-6). **Completed!**

One major experimental approach is to establish lentiviurs-based knockdown of PRMT5. As reported in the last two progress reports, we successfully identified two potent shRNA constructs that can knock down PRMT5. We also successfully established stable cell lines that can inducibly express shRNA to knock down PRMT5. Since we encountered some problems when we used the pool of stable cell lines, we isolated individual LNCaP clones to knock down PRMT5. These individually isolated clones should allow us to study the role of PRMT5 *in vivo*. Results were presented in the 2013-2014 Annual Report.

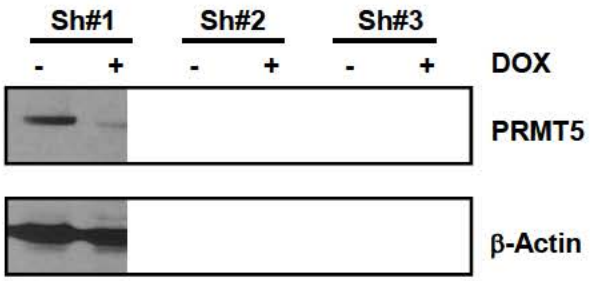

Figure 1. Establishment of stable DU-145 cell lines for inducible knockdown of PRMT5. Three individually isolated stable DU-145 cell lines (Sh#1-3) that can inducibly express PRMT5 short hairpin RNA was established. Cells were treated with doxycycline (DOX) (1 μg/ml) for 4 days, and total cell lysate was prepared for immunoblotting analysis of PRMT5. β-Atcin was used as a loading control.

To evaluate the effect of PRMT5 knockdown on radiation response in AR negative cell lines, we have also isolated individual clones from DU-145 (Fig. 1). Because both DU-145 and PC-3 behave very similarly and because both are AR negative cell lines, we will use DU-145 stable cell lines to conduct *in vivo* radiation experiments.

1b. Perform radiosensitization experiments by using the knockdown cell lines and by using PRMT5 small molecule inhibitor BLL3.3 (months 7-12). **Completed !**

We completed this task and demonstrated that knockdown of PRMT5 or inhibition of PRMT5 by BLL3.3 sensitized prostate cancer cells to ionizing radiation. This was reported in the 2012-2013 Annual Report

1c. Submit animal protocols for approval from Purdue University and USAMRMC. **Completed!**

We have completed the submission and approval of new animal protocols due to the expiration of our institute animal protocol.

1d. Perform in vivo radiosensitization experiments using prostate cancer cell xenograft tumors (LNCaP and DU-145) and analyze data (months 7-12). **Ongoing**.

Because we were limited by the lack of access to the Linear Accelerator in the Veterinary School at Purdue as we proposed in the original submission, we have approached Purdue University Center for Cancer Research, the College of Pharmacy and the department for the

support of purchasing an X-Ray irradiator. With their generous support, we have recently acquired a XRD-320 from Precision. The X-ray irradiator is housed in our new lab in the cancer center, to which we will relocate in the coming months. We anticipate that our move will happen in the middle of October, and we will start *in vivo* experiments using both LNCaP and DU-145 stable cell lines.

e. Analyze tumor tissues by immunohistochemistry (months 13-18). **Ongoing.**

We will complete the IHC analysis after we have finished up *in vivo* radiation experiments.

Task 2. Aim 2: To determine that targeting PRMT5 can sensitize recurrent (regrown) xenograft tumors to radiation and chemotherapy (Months 19-36)

2a. Isolate radiation-resistant prostate cancer sublines from DU-145 and PC-3 cells (months 19-24) **Completed and reported in the 2012-2013 Annual Report.**

2b. Perform radiosensitization and chemosensitizatio experiments using radiation-resistant sublines (Months 25-36). **Completed.**

We reported in the 2013-2014 Annual Report that inhibition of PRMT5 by the PRMT5 inhibitor BLL3.3 did not sensitize radioresistant sublines to three chemotherapeutic agents. As etoposide also induces double strand breaks, these results suggest that PRMT5 may utilize different mechanisms to sensitize prostate cancer cells to radiation. We have recently tested whether inhibition of PRMT5 by the BLL3.3 can sensitize both parental and radioresistant prostate cancer cells to radiation. As shown in Figure 2, inhibition of PRMT5 by BLL3.3 dramatically increased IR-induced cell death in LNCaP cells and to a lesser extent in DU-145 cells. However, BLL3.3 did not alter the response of isolated radioresistant sublines significantly (Fig. 2A and 2B). These results suggest that the radioresistance mechanism in these isolated radioresistant prostate cancer sublines may not involve PRMT5. Further research is needed to

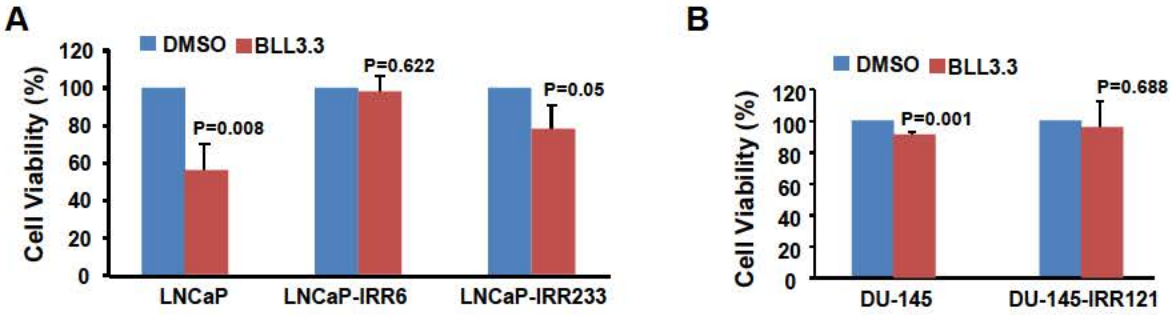

Figure 2. Effect of PRMT5 inhibition on radiation response of prostate cancer cells. A. Parental LNCaP cells and its derived radiation-resistant sublines IRR6 and IRR233 were seeded in 48-well plates and treated with the PRMT5 inhibitor BLL3.3 (10 μM) or DMSO while subjecting cells to fractionated ionizing radiation (FIR, 2 Gy/day) for 5 days. The inhibitor was freshly replaced every two days. Cell viability was measured using the MTT assay 24 hours after the last irradiation. **B.** Similar experiments were performed for parental DU-145 and its derived radiation-resistant subline IRR121.

elucidate the underlying radioresistance mechanism.

2c. Perform in vivo radiosensitization of recurrent xenograft tumors (Months 19-30). **Not started.**

Since our *in vitro* experiments suggest that PRMT5 may not be involved the acquisition of radioresistance in LNCaP and DU-145 sublines, we will not perform the proposed radiosensitization experiments. Instead, we will screen for additional mechanisms that may be involved. To conduct such an unbiased screen, we will use parental and radioresistant sublines to profile the expression of genes or miRNAs in a hope that we will discover some novel molecules involved.

2d. Analyze tumor tissues by immunohistochemistry (Months 31-36). **Not started.**

This subaim will not be pursued as we will not perform in vivo radiosensitization experiments with radioresistant sublines.

Task 3. Aim 3: To establish the clinical correlation between the expression level of PRMT5 and radioresistance and tumor recurrence (Months 1-36)

a. Submit IRB protocols to Purdue University, London Health Science Centre of the University of Ontario and USAMRMC (Months 1-6). **Completed.**

We have completed the submission of IRB protocols and we have received approvals.

b. Retrieve and review specimens for the proposed research (Months 7-12) **Ongoing.**

As reported in the 2013-2014 Annual Report, Dr. Chin and Dr. Moussa at the University of Western Ontario have encountered some difficulties to retrieve recurrent prostate cancer specimens archived many years ago. They have also found that many primary specimens were not available in their hospital. To solve this problem, I have contacted Dr. Richard Cho at Mayo Clinic Department of Radiation Oncology based on a published paper from Mayo Clinic [22]. Dr. Cho and Dr. Herrera Hernandex (pathologist) are now trying to see if they can retrieve some specimens from their archived samples.

3c. Prepare two slides from each specimens for IHC analysis (Months 13-18). ***See details in 3e.***

3d. perform IHC analysis and analyze data to establish the clinical correlation between PRMT5 expression and radioresistance and tumor recurrence (Months 19-36). ***See details in 3e.***

3d. Perform IHC analysis and analyze data to establish the clinical correlation between PRMT5 expression and radioresistance and tumor recurrence (Months 19-36) ***Ongoing***

As reported in the 2013-2014 Annual Report, we started to examine the expression level of PRMT5 in prostate cancer patients in case we will not be able to obtain enough number of recurrent prostate cancer specimens from patients who have failed radiotherapy. In collaboration with Dr. Jiaoti Huang at UCLA, we found that 60% of intermediate- and high-risk prostate cancer patients show moderate-to-strong expression whereas 40% of low-risk and 20% of normal control show similar extent of expression. These results strongly suggest that high expression of PRMT5 may indeed contribute to radioresistance. As this finding is very significant and interesting, we have tried to understand whether PRMT5 overexpression may contribute to

radioresistance at the molecular level. As reported in the 2013-2014 Annual Report, we have found that PRMT5 is an epigenetic regulator of androgen receptor (AR). This makes perfect sense that why PRMT5 inhibition can sensitize prostate cancer cells to radiation. Significantly, we have found that PRMT5 expression also correlates with AR expression in prostate cancer tissues (Fig. 3).

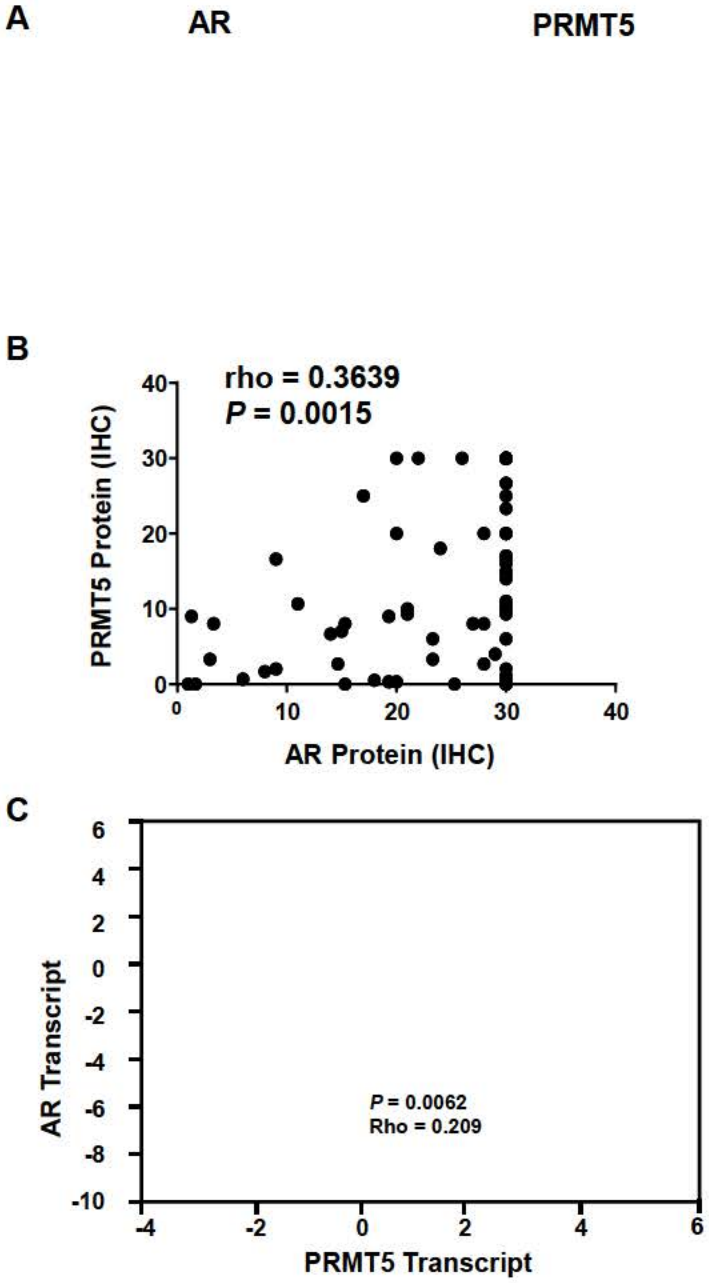

Figure 3. PRMT5 expression correlates with AR expression in prostate cancer tissues. A. Shown are representative immunohistochemical staining of AR (left) and PRMT5 (right) from serial sections. Note that those cells showing higher nuclear staining of PRMT5 also show strong staining for AR. **B.** Correlation of PRMT5 expression with AR in a prostate cancer tissue microarray. A tissue microarray containing 32 normal tissues, 20 prostate cancer tissues with Gleason score 6 and 20 prostate cancer tissues with Gleason score 7 and above was stained for AR and PRMT5 using immunohistochemical staining. Semi-quantitative analysis was performed and the Pearson correlation between the expression level of PRMT5 and AR was conducted. **C.** Correlation between PRMT5 transcript level and AR transcript level in prostate cancer tissues. The data were retrieved from Oncomine database, and those that have more 60 tissues were used for the Pearson correlation analysis.

Additional accomplishments relevant to proposed research

PRMT5 epigenetically regulates AR transcription. In the 2013-2014 Annual Report, we provided evidence that PRMT5 epigenetically regulates AR transcription. To understand how PRMT5 is recruited to the AR promoter, we have examined the interaction between PRMT5 and SP1, a major transcription factor to drive AR transcription in prostate cancer cells. As shown in Figure 4A, PRMT5 and SP1 was efficiently and reciprocally co-immunoprecipitated from LNCaP cells when either anti-PRMT5 or anti-SP1 antibody was used. Importantly, knockdown of SP1 not only dramatically decreased its own binding to the AR promoter (Fig. 4B) but also significantly reduced the binding of PRMT5 to the AR promoter (Fig. 4C). These results suggest that SP1 may recruit PRMT5 to the AR promoter for PRMT5 transcription.

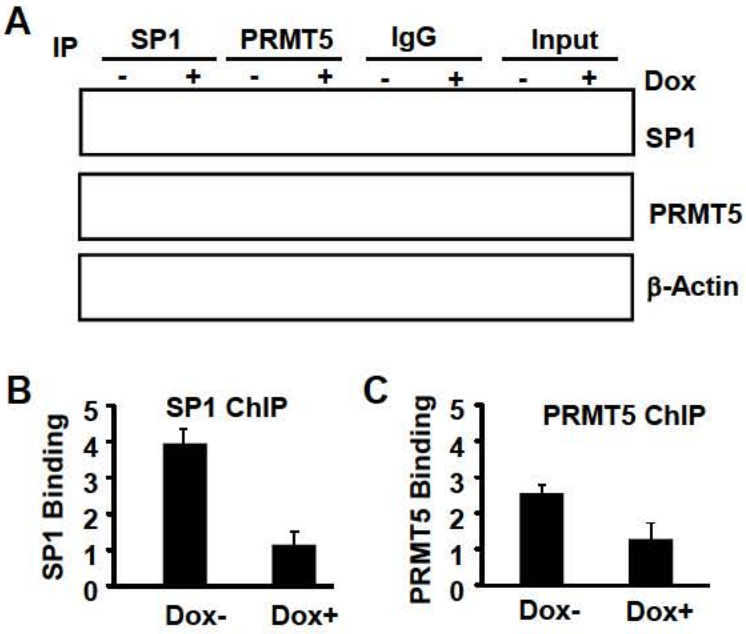

Figure 4. SP1 recruits PRMT5 to the AR promoter. A. Co-immunoprecipitation of PRMT5 and SP1 in LNCaP cells. LNCaP cells that can inducibly express SP1 shRNA were used to prepare total cell lysate, and 500 μg of total lysate was used for immunoprecipitation with SP1 antibody, PRMT5 antibody, or IgG control. Co-immunoprecipiated proteins were used for immunoblotting with PRMT5 or SP1 antibody. A significant enrichment of SP1 and PRMT5 was co-immunoprecipitated when compared with IgG controls in either doxycline (Dox+) treated or untreated (Dox-) cells. **B**.SP1 binds to the AR promoter. ChIP analysis was performed with anti-SP1 antibody in SP1 stable cell lines. The binding to the AR promoter was dramatically reduced by SP1 knockdown (Dox+). **C**. PRMT5 binding to the AR promoter. Similar ChIP experiment was performed except that anti-PRMT5 antibody was used for immunoprecipitation.

Regulation of PRMT5 expression by NF-Y in prostate cancer cells. In the 2013-2014 Annual Report, we showed that the PRMT5 proximal promoter region contains two binding sites for NF-Y, and that NF-Y appears to drive the transcription of PRMT5. Over the last year, we have made a significant progress in this direction and confirmed that NF-Y is a key transcription factor for PRMT5 transcription in prostate cancer cells. Importantly, we also discovered that the PKC signaling is a negative regulator of PRMT5. This is a significant finding given the recent finding that PKC mainly plays a negative role in controlling the growth of cancer cells [23]. In fact, we also found an inverse correlation between the expression level of several isoforms of PKC and PRMT5 in prostate cancer and lung cancer. A research article entitled "Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos

signaling in prostate cancer cells" published in Biochimica et Biophysica Acta [24] is included in this report as Appendix.

4. Key Research Accomplishments

- *Targeting PRMT5 does not sensitize radioresistant prostate cancer sublines to radiation.* We originally hypothesized that targeting PRMT5 would also sensitize radioresistant prostate cancer cells to radiation and chemotherapy given that higher expression level of PRMT5 is also maintained in isolated radioresistant sublines. In the 2013-2014 Annual Report, we reported that inhibition of PRMT5 by BLL3.3 did not sensitize isolated radioresistant LNCaP and DU-145 sublines to several chemotherapeutic agents including DNA damage-induced agents. In order to know whether inhibition of PRMT5 can sensitize these radioresistant sublines to radiation, we performed experiments with two LNCaP sublines and one DU-145 subline. Although we do see radiosensitization for parental LNCaP and DU-145 cells, inhibition of PRMT5 failed to sensitize these cells to radiation. Taken together, these results suggest that PRMT5 may not contribute to the radioresistance in these isolated radioresistant prostate cancer cells. A thorough investigation of potential mechanism is warranted for future research.
- *SP1 interacts with PRMT5 and recruits PRMT5 to the AR promoter*. We reported in the 2013-2014 Annual Report that PRMT5 epigenetically regulates AR transcription. This novel finding is significant and exciting given that PRMT5 is highly expressed in prostate cancer tissues and that AR is the driving force of prostate cancer development and progression. Since PRMT5 is an epigenetic enzyme that does not have DNA binding motif, it was unclear how PRMT5 is recruited to the AR promoter. To provide a mechanistic insight, we examined whether PRMT5 interacts with SP1, the major transcription factor for AR transcription in prostate cancer cells. We have successfully demonstrated that SP1 specifically interacts with PRMT5 and recruits PRMT5 to the AR promoter.
- *Transcriptional activation of PRMT5 by NF-Y is required cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells*. As described above, this work has been published in Biochimica et Biophysica Acta (Zhang et al, 1839:1330-1340, 2014). This represents the first study of PRMT5 transcriptional regulation.

5. Conclusion

Under the support of this prostate cancer idea development award, we have established several stable cell lines that can inducibly express PRMT5 shRNAs using LNCaP and DU-145 cell lines. These stable cell lines will be used for *in vivo* radiation experiments to evaluate the effect of PRMT5 knockdown on the response of xenograft tumors to radiation. We have successfully isolated radioresistant sublines from LNCaP and DU-145, and we have examined the effect of PRMT5 targeting on chemosensitivity, and found that inhibition of PRMT5 by BLL3.3 does not sensitize both radioresistant sublines and their parental LNCaP and DU-145 cells to three chemotherapeutic agents (docetaxel, cisplatin and etoposide) as reported in the 2013-2014 Annual Report. Unexpectedly, we have also found that PRMT5 inhibition did not sensitize these radioresistant cells to ionizing radiation. These results suggest that additional mechanisms may be involved in radioresistance and chemoresistance in these isolated radiation-resistant sublines. As we have generated these precious radiation-resistant sublines, we will take full advantage of these sublines to conduct systematic investigation of the underlying mechanisms.

Since our collaborators at the University of Western Ontario have encountered some difficulties to retrieve specimens from patients who had recurrence after radiotherapy, we have started alternative approaches to test our hypothesis. We have examined PRMT5 expression in a prostate cancer tissue microarray at UCLA and found that PRMT5 is highly expressed in intermediate- and high-risk prostate cancer tissues when compared with low-risk prostate cancer tissues and normal tissues. This finding is consistent with the clinical observation that high recurrence rate was reported in intermediate- and high-risk patients. Importantly, we have found that PRMT5 epigenetically regulates AR transcription. Our new results further suggest that the recruitment of PRMT5 to the AR promoter is likely mediated through its physical interaction with SP1, the major transcription factor involved in AR transcription in prostate cancer cells. In conclusion, our new results collectively support our overall hypothesis that PRMT5 overexpression in prostate cancer tissues contributes to radioresistance in primary tumors.

Because PRMT5 is highly expressed in intermediate- and high-risk cancer patients and because radiation appears to induce PRMT5 expression, we started to investigate how PRMT5 expression is regulated two years ago. We have found that NF-Y is a transcription factor for PRMT5 transcription and cell growth in prostate cancer cells. More importantly, molecular analysis has identified the PKC/c-Fos signaling as a negative regulator of the NF-Y/PRMT5 axis in prostate cancer cells. Consistent with this, the transcript level of several isozymes of PKC inversely correlates with the transcript level of PRMT5 in prostate cancer tissues. This finding for the first time links the PKC signaling to NF-Y and PRMT5, both of which are considered oncoproteins. This work was published Biochim Biophys Act (2014 Nov). In fact, a recent genetic study from the Newton group provides evidence supporting that PKC isozymes generally function as tumor suppressors rather than oncogenes. Therefore, restoring the PKC activity rather than inhibiting the PKC activity should be pursued as a direction of future drug development.

6. Publications, Abstracts, and Presentations

(1) Publications

a. Zhang HT, Zhang D, Zha ZG and Hu CD. Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells. Biochim Biophys Acta, 1839:1330-1340 (2014)

b. Suarez CD, Deng X, and Hu CD Targeting CREB inhibits radiation-induced neuroendocrine differentiation and increases radiation-induced cell death in prostate cancer cells. *Am J Cancer Res*, 4:850-861 (2014)

c. Hu CD, Choo R, and Huang J. Neuroendocrine differentiation in prostate cancer: a mechanism of radioresistance and treatment failure. Front Oncol, 5:90. Doi: 10.3389/fonc.2015.00090 (2015).

(2) Presentations

a. Targeting PRMT5 for prostate cancer ratiosensitization
   Place: Jinan University College of Medicine
   Date: December 29, 2014

b. Advances in prostate cancer diagnosis and treatment- A comparative analysis between China and America
   Place: Tongling First People's Hospital
   Date: January 5th, 2015

c. Mechanisms and targeting of therapy-resistant prostate caner
   Place: Purdue-IU Cancer Retreat
   Date: May 1st, 2015

7. Inventions, Patents and Licenses

None

8. Reportable Outcomes

None

9. Other Achievements

a. Establishment of stable cell lines that inducibly express PRMT5 shRNA from LNCaP and DU-145. These cell lines will be used for proposed *in vivo* experiments.

b. Gyeon Oh, a graduate student who partially worked on the project graduated with MS degree in May 2015. She is now studying for her Ph.D. at University of Kentucky.

c. Huantin Zhang, a visiting graduate student who worked on the transcriptional regulation of PRMT5 has published his work in BBA. He returned to his home institution to continue his study at Jinan University. He was awarded Ph.D. in July, 2015.

d. Yihang Wu, a visiting professor from Jiliang University, China was studying in my lab and participating in the project. He received training in molecular biology and returned to his home institution on August 17, 2015.

e. Training of rotation students and recruitment of two new graduate students: three graduate students (Sarah Kelsey, Lama Abdullah Alabdi and Jake Owens) received training during their rotations. Jake Owens and Sarah Kelsey have joined the lab and are partially working on the project.

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Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells

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ABSTRACT

Protein arginine methyltransferase 5 (PRMT5) symmetrically methylates arginine residues of histones and non histone protein substrates and regulates a variety of cellular processes through epigenetic control of target gene expression or post translational modification of signaling molecules. Recent evidence suggests that PRMT5 may function as an oncogene and its overexpression contributes to the development and progression of several human cancers. However, the mechanism underlying the regulation of PRMT5 expression in cancer cells remains largely unknown. In the present study, we have mapped the proximal promoter of PRMT5 to the 240 bp region and identified nuclear transcription factor Y (NF Y) as a critical transcription factor that binds to the two inverted CCAAT boxes and regulates PRMT5 expression in multiple cancer cell lines. Further, we present evidence that loss of PRMT5 is responsible for cell growth inhibition induced by knockdown of NF YA, a subunit of NF Y that forms a heterotrimeric complex with NF YB and NF YC for function. Significantly, we have found that activation of pro tein kinase C (PKC) by phorbol 12 myristate 13 acetate (PMA) in LNCaP prostate cancer cells down regulates the expression of NF YA and PRMT5 at the transcription level in a c Fos dependent manner. Given that down regulation of several PKC isozymes is implicated in the development and progression of several human cancers, our findings suggest that the PKC c Fos NF Y signaling pathway may be responsible for PRMT5 overexpression in a subset of human cancer patients.

1. Introduction

Protein arginine methyltransferase 5 (PRMT5), a type II methyl transferase that symmetrically methylates arginine residues of histones and non histone protein substrates [1,2], regulates a variety of cellular processes by epigenetic regulation of target gene expression and by post translational modification of critical signaling molecules [1]. Recently, several studies have shown that PRMT5 is overexpressed in human cancers such as lung cancer [3,4], ovarian cancer [5], colorectal cancer [6], breast cancer [7], melanoma [8], leukemia and lymphoma [9,10], and glioblastoma [11]. The overexpression of PRMT5 correlates with disease progression and poor prognosis. Importantly, these studies also present evidence that silencing PRMT5 expression in these cancer cells inhibits cell proliferation and/or induces apoptosis, suggesting that PRMT5 overexpression in cancer cells plays an important role in the development and progression of human cancers. However, how PRMT5 expression is transcriptionally regulated in cancer cells has not yet been investigated.

Nuclear transcription factor Y (NF Y) is an important transcription factor that is highly conserved across the species [12 14]. NF Y is com posed of three subunits, NF YA, NF YB and NF YC, and functions as a heterotrimeric complex to bind the CCAAT box in promoter regions to regulate gene transcription. CCAAT boxes are usually positioned in either orientation between −60 and −100, and are present in almost 30% of human promoters, particularly those that drive expression of on cogenes in human cancers [15 17]. In addition, NF Y binding sites over lap with binding sites of several other transcription factors, such as SP1, E2F1, GATA, and c Fos, to cooperatively regulate cell growth [12,15,18]. The NF Y transcriptional activity can be modulated by increasing DNA binding to the CCAAT boxes [19,20] or by increasing expression of the NF YA subunit [12,21 23]. However, whether the cancer signaling regulates NF YA expression remains unknown.

Abbreviations: PRMT5, Protein arginine methyltransferase 5; NF-Y, Nuclear transcription factor Y; PKC, Protein kinase C; AP-1, Activator protein-1; PMA, Phorbol 12-myristate 13-acetate; GFX, Bisindolylmaleimide I; TCL, Total cell lysate; CCNA2, Cyclin A2; Dox, Doxycycline; SC, Scrambled control; shRNA, Short hairpin RNA; BrdU, Bromodeoxyuridine; indel, Insertion–deletion; SNPs, Single nucleotides polymorphisms; WT, Wild-type

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Protein kinase C (PKC) is a family of serine/threonine protein kinases that regulates a wide range of cellular processes [24]. PKC isozymes can be classified into three groups including calcium dependent "classical" cPKCs (α, βI, βII and γ), calcium independent "novel" nPKCs (δ, ε, η and θ), and calcium independent "atypical" aPKCs (ζ and ι/λ). Classical and novel PKC isozymes, but not atypical PKC isozymes, can be activated by diacylglycerol (DAG) and phorbol 12 myristate 13 acetate (PMA). Although it is generally thought that most PKC isozymes are overexpressed in human cancers and promote cellular transformation, proliferation, and migration, the opposite effects have also been reported [24]. This is exemplified by the use of prostate cancer cells as a model system to study distinct roles of PKC isozymes in apo ptosis in prostate cancer cells [25], in which treatment of LNCaP, but not DU 145 and PC 3 cells, with PMA induces apoptosis [26]. Consistent with their differential roles in cell based studies, the expression level of several PKC isozymes in some human cancers inversely correlates with the aggressiveness of the disease [27,28]. However, the mechanism by which down regulation of PKC isozymes regulates cancer cell growth remains unknown.

Activator protein 1 (AP 1) is a family of dimeric transcription factors which includes c Jun and c Fos [29]. AP 1 was discovered as a complex of c Fos/c Jun that can be induced by serum and PMA [30 32]. Although activation or overexpression of AP 1 proteins is implicated in the devel opment and progression of many human cancers, distinct roles of AP 1 proteins have also been observed [29,33,34]. For example, reduced expression of c Fos and c Jun has been observed in a subset of human prostate cancer patients [35 38], though the clinical significance of reduced AP 1 protein expression remains unclear. Recently, we have demonstrated that c Jun acts as a transcriptional repressor of the andro gen receptor (AR) signaling, and that silencing c Jun promotes the growth of both androgen dependent LNCaP cells and castration resistant C4 2 cells [39], providing evidence that down regulation of c Jun expression in a subset of human prostate cancer patients may pro mote disease progression by enhancing the AR signaling. In the present study, we demonstrate that NF Y is a major transcription factor to drive PRMT5 transcription in several cancer cell lines, and knockdown of NF YA leads to down regulation of PRMT5 expression and suppression of cell growth. Further, we show that PMA treatment in LNCaP cells down regulates the expression of NF YA and PRMT5 in a PKC and c Fos dependent manner.

2. Materials and methods

2.1. Cell culture and treatment

The prostate cancer cell lines LNCaP and PC 3 cells were cultured as described previously [40,41]. Lung cancer cell line A549 was kindly pro vided by Wanqing Liu, and cells were cultured in F K12 medium con taining 10% fetal bovine serum and 1% penicillin/streptomycin. PMA was purchased from Sigma (P 1585), and bisindolylmaleimide I (GF109203X, GFX), a pan PKC inhibitor, was purchased from Tocris Bioscience (a gift of the Val Watts lab). For PMA treatment, cells were seeded into 6 cm dishes for 24 h (approximately 80 90% confluence), and then treated with different doses of PMA for the indicated times in the presence or absence of GFX.

2.2. Plasmid construction

Two distinct types of the PRMT5 promoters (−3461/+75 bp and −3474/+75 bp) were amplified from LNCaP cell genomic DNA by PCR with Phusion High Fidelity DNA Polymerase (NEB) using primers 5′ CGGGGTACCCTGGGCACAACTAGGGCAGAGAAC 3′ and 5′ GAAGAT CTTCCACGCCGGGATTCCTTGATAC 3′. The PCR products were then cloned into pGL4.10 [*luc2*] Basic Vector (Promega). To construct a series of luciferase reporter genes (A1: −1723/+75, A2: −1156/+75, A3: −459/+75, A4: −323/+75, A5: −240/+75, B1: −1736/+75, B2: −1169/+75, B3: −472/+75, B4: −323/+75, B5: −240/+75, B6: −68/+75, B7: +8/+75), the same methods were used for PCR ampli fication by using two types of PRMT5 promoters as templates. For muta genesis, nucleotide substitutions in putative binding motifs were introduced by ligation PCR [42]. The expression plasmids pFLAG c Fos and pFLAG c Jun were previously constructed [39,43,44]. The cDNA encoding PRMT5 was amplified by PCR using primers 5′ CTGAATTCGG ATGGCGGCGATGGCGGT 3′ and 5′ GCCTCGAGAGAGGCCAATGGTATA TGAGCG 3′ and cloned into pCMV Myc vector (Clontech). All plasmid constructs were verified with DNA sequencing.

2.3. Luciferase reporter gene assay

Prostate cancer cells were plated in 12 well plates at a density of 2 x 10^5/well, and A549 cells were plated at a density of 1×10^5/well. After 24 h, 1 μg of a short hairpin RNA (shRNA) plasmid targeting NF YA was transiently co transfected with 0.5 μg of a PRMT5 reporter gene, along with 0.1 μg of pRL TK (Promega) by FuGENE HD or FuGENE 6 (Promega). Forty eight hours after transfection, Firefly and Renilla luciferase activities were determined by a TopCount NXT microplate luminescence counter (Packard) using dual luciferase Reporter Assay Kit (Promega) according to the manufacturer's instruction with minor modifications as described previously [43,44].

2.4. Immunoblotting

Preparation of total cell lysate (TCL) and immunoblotting were per formed as described before [41]. Densitometric quantification was per formed with Image J software (NIH, Rockville, MD, USA). The antibodies used for immunoblotting analysis were: anti β actin (A1978, Sigma), anti NF YA (H 209, sc 10779, Santa Cruz) [45], anti c Jun (H 79, sc 1694, Santa Cruz), anti c Fos (H125, sc 9202, Santa Cruz), anti PRMT5 (07 405, Millipore), anti FLAG M2 (F3165, Sigma), anti Myc (631206, Clontech), and anti cyclin A2 (CCNA2, BF683, Cell Signaling). Secondary HRP conjugated antibodies were purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK).

2.5. RNA isolation and quantitative real time PCR (qRT PCR)

Total RNA was isolated from cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instruction and verified for integrity by agarose gel electrophoresis. One microgram of RNA was used for reverse transcription using random primers (100 ng) and MMLV reverse transcriptase (Promega). The mRNA level of PRMT5, NF YA, NF YB, NF YC and GAPDH was quantified using qRT PCR with gene specific primers. PRMT5 forward, 5′ CAGAGAAGGAGT TCTGCTCCTAC 3′ and PRMT5 reverse, 5′ ATGGCCTGCTGGTACTGAGA GT 3′; NF YA forward, 5′ CTGTGACACTACCAGTGGCAG 3′ and NF YA reverse, 5′ TGCCTCCTCTTAAGAATACGG 3′; NF YB forward, 5′ GCAA GTGAAAGGTGCCATCAAGAG 3′ and NF YB reverse, 5′ CTGCTCCACCAA TTCCCTTTTCTC 3′; NF YC forward, 5′ GAACTGAAACCTCCAAAGCGTC 3′ and NF YC reverse, 5′ TGTGCGATGATGATCTGCCCAG 3′. GAPDH for ward, 5′ CTGACTTCAACAGCGACACC 3′ and GAPDH reverse, 5′ CCCTGT TGCTGTAGCCAAAT 3′. qRT PCR was performed with SYBR@GREEN PCR Master Mix (Roche) by using a ViiA7 Real Time PCR system (Applied Biosystems) for 40 cycles. The relative expression of each individual gene was normalized to GAPDH and was calculated using the compara tive $2^{-\Delta\Delta CT}$ method [46].

2.6. Chromatin immunoprecipitation (ChIP)

Cells cultured in 10 cm dishes were cross linked with 1% formalde hyde for 10 min and then stopped by adding 125 mM glycine. Chroma tin from two dish cells was sheared by a Branson Digital Sonifier 250 to an average size of approximately 0.5 kb in 1 ml immunoprecipitation (IP) buffer (50 mM Tris·Cl, pH 7.4, 0.5% NP 40, 1% Triton X 100,

150 mM NaCl, 5 mM EDTA, and 0.5 mM DTT). The sheared chromatin (DNA protein complexes) was incubated with anti NF YA (G 2, sc 17753X, Santa Cruz) [47], or the control IgG (sc 2025, Santa Cruz) at 4 °C for overnight and the DNA protein complexes were recovered by protein G agarose beads (Santa Cruz, sc 2002). The immunoprecipitated DNA was isolated by 10% Chelex 100 using the fast ChIP method [48], and then subjected to qRT PCR. The relative fold enrichment was calculated by normalizing to IgG control. A non target region in the PRMT5 distal promoter and a region containing a validated NF Y binding site in the CCNA2 promoter were amplified from the same IP sample, and used as negative control and positive control, respectively. The primers used for ChIP are listed as follow: the region containing two NF Y binding sites in the PRMT5 proximal promoter (5′ CACTGTTTCTCTCCGTGATGGTAC 3′ and 5′ GCGTCTGCCACAGCTCCCGAAC 3′); and a non target region in the PRMT5 distal promoter (5′ CTGGGCACAACTAGGGCAGAGAAC 3′ and 5′ TTAGTAGAGACGGGGTTTCAC 3′); the region containing one validated NF Y binding site in the CCNA2 promoter (5′ GCCCCTGCTCAGTT TCCTTTG 3′ and 5′ CGGCGGCTGTTCTTGCAGTTCA 3′).

2.7. Lentivirus production and establishment of stable cell lines

For the construction of shRNA expressing plasmids, the pLKO Tet On inducible lentiviral RNAi system was used [49]. Several targeting sequences were selected from the RNAi Consortium (Sigma) as follow: NF YA (shYA#1), 5′ CCATCGTCTATCAACCAG TTA 3′ (TRCN0000014930); NF YA (shYA#2), 5′ CCATCATGCAAG TACCTGTTT 3′ (TRCN0000014932); and c Fos, 5′ GCGGAGACAG ACCAACTAGAA 3′ (TRCN0000273941). Scrambled control (SC), 5′ AACAAGATGAAGAGCACCAA 3′, was used as a negative control for all knockdown experiments. Annealed oligonucleotides were cloned into pLKO Tet On. To generate viral particles, HEK 293 T cells were cultured in a 10 cm dish without antibiotics for 24 h, and then co transfected with 2 μg of pLKO.1 Tet On shRNA vector, 1.5 μg of pHR' CMV ΔR8.2Δvpr packaging plasmid, and 0.5 μg of pHR' CMV VSVG envelope plasmid using FuGENE HD reagent. The supernatant containing viruses was harvested 3 days post transfection, and then filtered through a 0.45 μm filter to remove cell debris. Prostate cancer cells and lung cancer cells were then infected by applying 6 ml viral supernatant in 10 ml complete medium. Polybrene was added to a final concentration of 8 μg/ml to facilitate the infection. Cells were selected with 2 μg/ml of puromycin (for PC 3, 3.5 μg/ml) for 3 days for stable integration of the shRNA plasmids, and surviving cells were maintained in the presence of 1 μg/ml of puromycin. To knock down NF YA or c Fos, cells were induced with 1 μg/ml of doxycycline (Dox) for at least 3 days.

2.8. Cell growth analysis and Trypan blue exclusion assay

LNCaP and PC 3, or A549 stable cell lines were seeded in six well plates in triplicate at a density of 1×10^5 cells/well or 2×10^4 cells/well, respectively. Cells were then induced with or without Dox (1 μg/ml) for various times, and medium and Dox were changed every 3 days during culture. The number of viable and dead cells from each well was determined by Trypan blue staining. To determine the effect of NF YA knock down on cell proliferation, the indicated stable cell lines were seeded and grew on coverslips in six well plates at a cell density of 1×10^5 cells/well or 2×10^4 cells/well, followed by treatment with or without Dox (1 μg/ml) for 84 h. Bromodeoxyuridine (BrdU, Calbiochem Cat#QIA58) was then added to each well for incubation of another 8 h and cells were processed as described previously [39]. For quantification of BrdU incorporated cells, at least 1000 cells from 10 fields were counted for each cell line under a Nikon TE2000 U inverted fluorescence microscope. Fluorescent images were taken at 200× magnification and the percentage of BrdU positive cells was shown.

2.9. Statistical analyses

Statistical analyses were performed with the GraphPad Prism 6 Software (Graphpad Software, San Diego, CA, USA). Briefly, Student's *t* test was used to compare means of two different groups, while one way analysis of variance (ANOVA) was used for multiple group comparison, followed by Tukey's post hoc test or Dunnett's test. Two way ANOVA was used to compare the means of two independent variables, followed by Tukey's post hoc test. All data were expressed as mean ± SEM, and *p* values less than 0.05 between groups were considered statistically significant. To analyze the correlation between the expression of PRMT5 and NF YA in prostate cancer, we searched the Oncomine database (www.oncomine.org) and included each study that has more than 60 samples. A total of six independent studies met this criterion, and the results from these studies were pooled for correlation analysis. For each pair, the statistic Q was calculated to test the homogeneity of effect sizes across studies [50]. It turns out that, for each pair, the effect sizes across studies are not homogeneous (all with p value < 0.0001). Therefore, we employed a random effects model for the meta analysis of each pair [51].

3. Results

3.1. Identification of the proximal promoter of PRMT5

To investigate how PRMT5 expression is transcriptionally regulated, we cloned a 3.5kb PRMT5 promoter from LNCaP cells and found that there were two distinct types of promoters that harbor six single nucleotide polymorphisms (SNPs) and one 13 bp insertion/deletion polymorphism (indel) within 1.8 kb (Fig. 1A). To know whether these SNPs may impact the promoter activity, we used the 1.8 kb of the promoter to construct a series of truncated luciferase reporter genes (Fig. 1A). Transfection of these reporter genes into LNCaP cells resulted in at least a 7 fold increase in the promoter activity when compared with the vector control, with the B3 showing the highest activity (Fig. 1B). Similar results were obtained in PC 3 cells (Fig. 1C). However, mutations of all SNPs did not show any significant impact on the reporter gene activity (data not shown). Taken together, these results suggest that these SNPs have negligible effect on the 1.8 kb promoter activity.

To identify a proximal promoter region, we constructed two other reporter genes (B6: −68/+75; B7: +8/+75) (Fig. 1D) and found that further deletions (B6 and B7) dramatically decreased the reporter gene activity in LNCaP cells (Fig. 1D), indicating that the region −240 to +75 is critical for the PRMT5 promoter activity. Similar results were observed in PC 3 cells (Fig. 1D). Since PRMT5 expression is also required for the growth of lung cancer cells (A549) [3], we transfected these reporter genes into A549 cells and observed that the reporter gene activity of B5 in A549 was 2 fold higher than that in LNCaP and PC 3 cells, though a comparable reporter gene activity of B6 and B7 was observed in all three cell lines (Fig. 1D). These results demonstrate that the proximal −240 region is important for PRMT5 transcription in a cell context dependent manner.

3.2. The two inverted CCAAT boxes are critical for the proximal promoter activity of PRMT5

We next used AliBaba2.1 and TFSEARCH online software to search for putative *cis* regulatory elements and identified one consensus GATA binding site for GATA binding, one GC box for SP1 binding, and three identical inverted CCAAT boxes for NF Y binding in the proximal promoter region (Fig. 2A). In order to determine whether these putative binding sites contribute to the proximal promoter activity, we mutated these consensus motifs by site directed mutagenesis (Fig. 2B), and examined their activities by using the luciferase reporter gene assays. In LNCaP cells, mutation of Y1 or Y2 (from CCAAT to CAGAA) [52], decreased the reporter gene activity by 33% and 21%, respectively

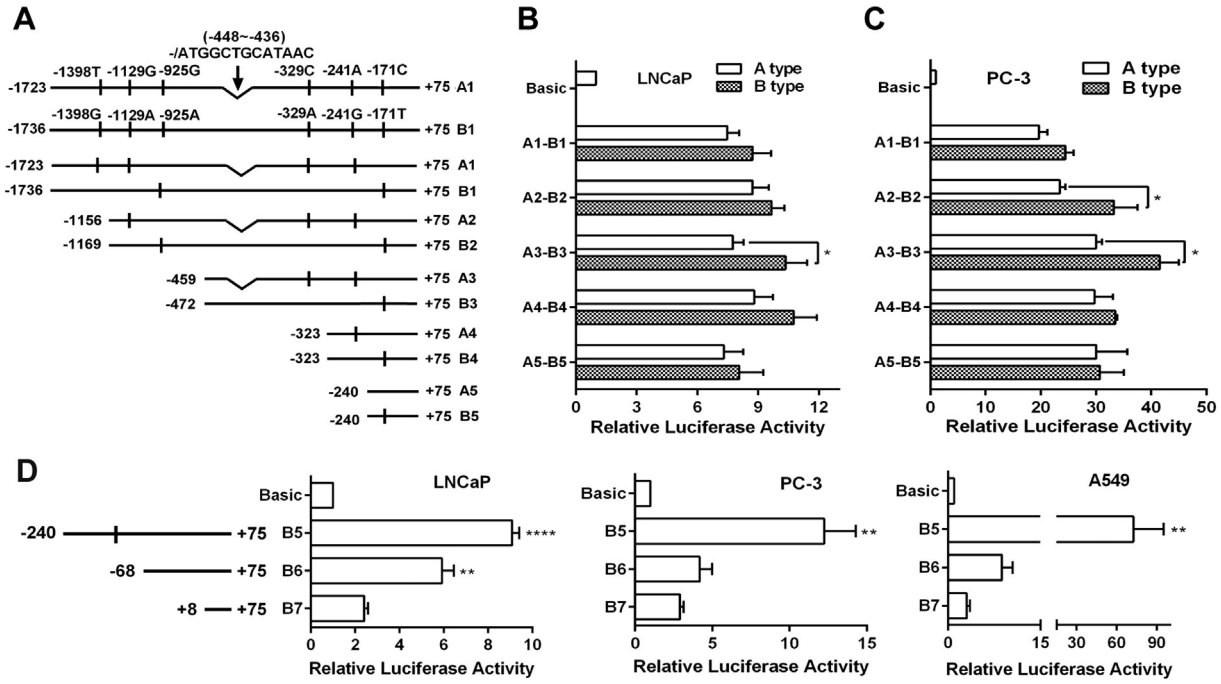

Fig. 1. Identification of the proximal promoter of PRMT5. (A) Two types of PRMT5 promoters cloned from LNCaP genomic DNA with indicated SNPs and an indel, as well as a series of 5′-truncated promoters were used to construct luciferase reporter genes. (B and C) The indicated reporter genes in A were co-transfected with pRL-TK into LNCaP and PC-3 cells for 24 h for measurement of the luciferase activities. Results were obtained from at least three independent experiments in triplicate, and were normalized to the vector control (Basic). (*$p < 0.05$; Student's t test). (D) Luciferase activities of 5′-truncated reporter genes (B6 and B7) in LNCaP, PC-3 and A549 cells. Results from four to six independent experiments are presented as mean ± SEM. Statistical significance (**$p < 0.01$ and ****$p < 0.0001$) was determined when compared with B7 by one-way ANOVA followed by Dunnett's test.

(Fig. 2C). Significantly, mutations of both NF Y binding sites resulted in 70% reduction in the reporter gene activity. Contrary to the two CCAAT box binding sites, single mutation introduced into the SP1 (GGGCGG to GGAAAG) or GATA (GATA to GCAA) binding site, which was demonstrated previously to abolish their binding [53,54], increased the promoter activity by 36% or 27%, respectively (Fig. 2C). However, mutation of both SP1 and GATA binding sites did not show any further increase in the promoter activity. Similar effect of mutations in NF Y sites was observed in PC 3 (Fig. 2D) and A549 cells (Fig. 2E), though single mutation of the first NF Y site (Y1) had a more profound effect compared with the second NF Y site (Y2). These results suggest that the two NF Y binding sites may positively regulate PRMT5 transcription in all three cell lines whereas the SP1 and GATA binding sites may negatively regulate PRMT5 transcription in LNCaP cells but not in PC 3 and A549 cells. To know how these binding sites cooperatively contribute to the PRMT5 promoter activity, we mutated these binding sites in combination (Fig. 2B), and observed an overall inhibitory effect on the luciferase reporter gene activity, which was similar to the effect of mutations in the first two NF Y binding sites (mY1,2). Note that a third NF Y binding site (Y3) is located at +42, however, mutation of Y3 did not decrease the reporter gene activity in all three cell lines. Instead, a slight increase was observed (Fig. 2F H). When all three NF Y binding sites were mutated, a comparable suppression of the reporter gene activity to that with Y1/Y2 mutated was observed in all three cell lines (Fig. 2F H). Taken together, these results suggest that the first two putative NF Y binding sites are the major *cis* regulatory elements to drive PRMT5 transcription.

3.3. NF Y regulates PRMT5 expression in LNCaP cells via binding to the two CCAAT boxes

Unlike NF YB and NF YC, whose expression is relatively stable, NF YA is the limited subunit for specific binding to CCAAT boxes in cells [12,21 23]. To confirm the role of NF Y in PRMT5 transcription at the endogenous level, we established two stable cell lines that inducibly express shRNAs targeting two different sequences in the coding region of NF YA to evaluate the effect of NF YA knockdown on PRMT5 expression. As shown in Fig. 3A, the two shRNAs knocked down the expression of NF YA S, the shorter isoform of NF YA that is predominantly expressed in LNCaP cells, by more than 65%. The reduction of PRMT5 expression at protein level was similar to that of NF YA. We confirmed that the expression of a well known NF Y target gene CCNA2 was also reduced, demonstrating the specificity of the two NF YA shRNAs. Since the shYA#1 showed higher knockdown efficiency in LNCaP, it was chosen for the following experiments. We found that knockdown of NF YA decreased the PRMT5 mRNA level (Fig. 3B), suggesting that the reduction of PRMT5 by NF YA knockdown likely occurs at the transcriptional level. Transient knockdown of NF YA significantly inhibited the WT reporter gene activity, but had no effect on the mutant reporter gene activity (Fig. 3C), suggesting that the two CCAAT boxes in the proximal promoter region likely mediates the effect of NF Y on PRMT5 transcription. We next performed ChIP assays and confirmed that NF YA bound to the region containing the two CCAAT boxes (P2 in Fig. 3D), but not the distal promoter region that does not contain CCAAT box (P1 in Fig. 3D). As a positive control, NF Y also bound to the proximal promoter of CCNA2 [55]. These results demonstrate that NF Y indeed binds to the two CCAAT boxes in the proximal promoter of PRMT5 and regulates PRMT5 transcription in LNCaP cells. To know whether NF Y may regulate PRMT5 expression in human prostate cancer tissues, we searched Oncomine database and found that there was a strong positive correlation between the transcript level of NF YA and PRMT5 (Fig. 3E), as evidenced by a meta analysis from six independent studies. This result further supports our finding that NF Y regulates PRMT5 expression in prostate cancer cells.

3.4. NF Y regulation of PRMT5 expression is required for prostate cancer cell growth

Given that NF Y is critical for PRMT5 expression in several cancer cell lines, we next sought to determine the importance of NF Y regulation of

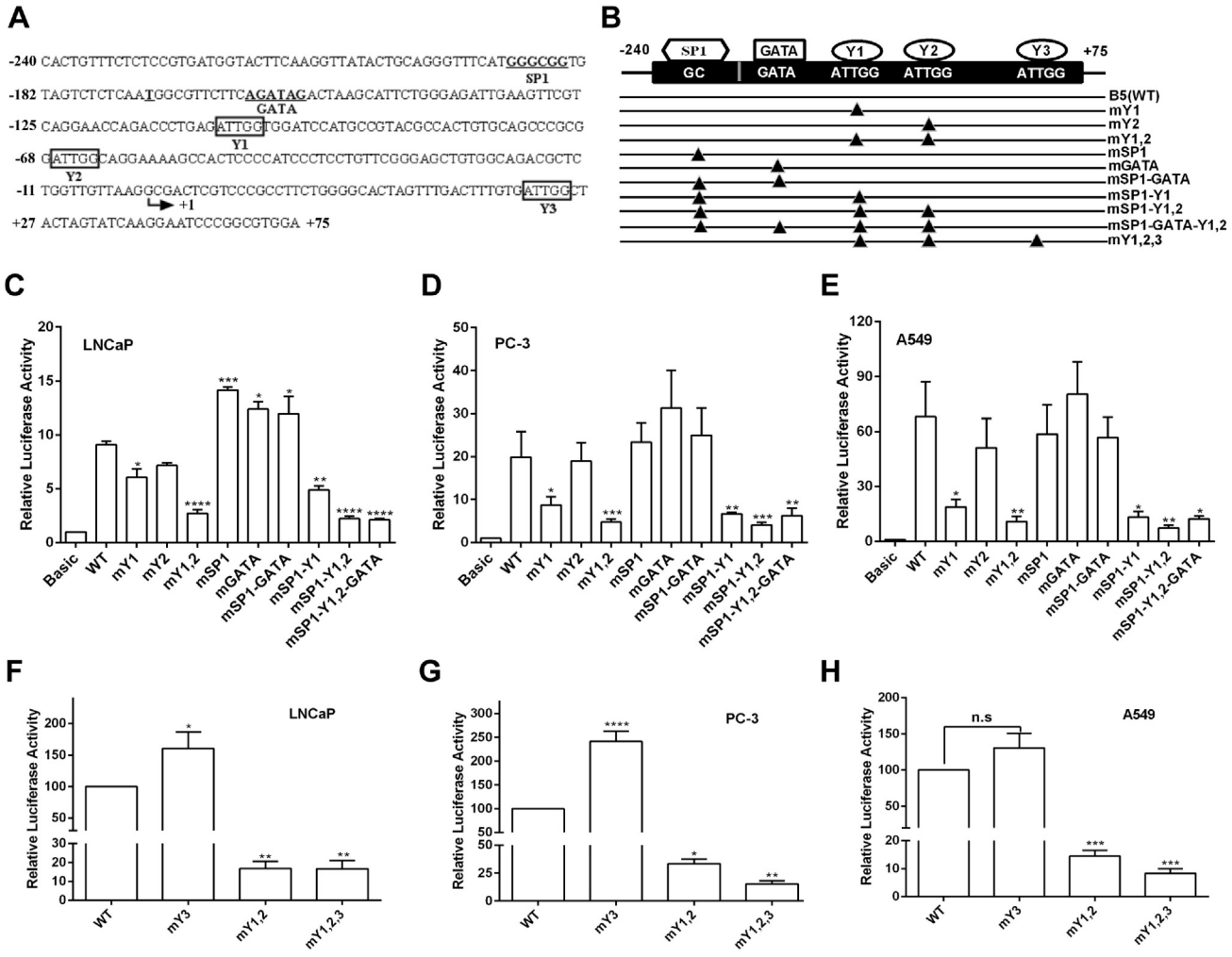

Fig. 2. The two CCAAT boxes are critical for the proximal promoter activity of PRMT5. (A) Sequences of the proximal promoter region from −240 to +75 with predicted *cis*-regulatory elements. The transcription start site was indicated by arrow. Y1, Y2, or Y3 indicates the first, second or third NF-Y binding site. (B) Illustration of a series of B5-based luciferase reporter gene constructs. Triangle indicates the corresponding *cis*-regulatory element was mutated. (C–E) CCAAT boxes are critical for luciferase activity driven by the PRMT5 promoter. The luciferase activity of the indicated reporter gene constructs in B was determined in the indicated cancer cell lines. (F–H) The third NF-Y binding site has little effect on the PRMT5 promoter activity. The indicated luciferase reporter gene was co-transfected with pRL-TK into LNCaP (F), PC-3 (G) and A549 (H) cells for 24 h, and the relative luciferase activity was determined. Results in C–H were from at least three independent experiments, and were normalized to the vector control and are presented as mean ± SEM. Statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$) was determined when compared with WT (wild-type) by one-way ANOVA followed by Dunnett's test.

PRMT5 expression in cell growth. Using the two shRNA constructs, we were able to establish a stable cell line by using A549 to knockdown NF YA by 50%, accompanied by a 39% reduction in PRMT5 expression (Supplementary Fig. S1A). However, the two shRNAs did not exhibit acceptable knockdown efficiency in PC 3 (Supplementary Fig. S1B). We then examined the effect of NF YA knockdown on cell growth and cell death in LNCaP and A549. Knockdown of NF YA inhibited cell growth in LNCaP and A549 cells (Fig. 4A and B). The inhibition of cell growth in both LNCaP and A549 by NF YA knockdown was attributable to the inhibition of cell proliferation (Fig. 4C and D; Supplementary Fig. S1C and D) and the induction of cell death (Fig. 4E and F), in agreement with previous findings that NF Y plays a role in regulating cell proliferation and cell death [12]. Because NF Y may influence growth of these cancer cells by controlling expression of many other genes [12,15,17], we next performed a PRMT5 rescue experiment to determine to what extent PRMT5 down regulation is responsible for cell growth inhibition induced by NF YA knockdown. As shown in Fig. 4G and H, transient expression of PRMT5 partially rescued cell growth inhibition only in LNCaP cells, but not in A549 cells. Taken together, these results suggest that the regulation of cell growth by NF Y may be partially mediated through up regulation of PRMT5 expression in a cell context dependent manner.

3.5. The PKC signaling negatively regulates PRMT5 expression in LNCaP cells

We next searched for possible cell signaling that may regulate PRMT5 expression in LNCaP cells by treating cells with various protein kinase inhibitors or agents that activate cell signaling pathways, and observed that treatment of cells with PMA resulted in a dramatic decrease of PRMT5 expression in a dose and time dependent manner (Fig. 5A and B). Interestingly, NF YA expression was similarly inhibited (Fig. 5A and B). Significantly, the mRNA level of PRMT5 (Fig. 5C) and NF YA, but not NF YB and NF YC (Fig. 5D), was inhibited by PMA treatment as well. Because PMA induced PKC activation contributes to cell growth inhibition and apoptosis in LNCaP cells [26], we examined whether inhibition of PKC can restore the expression of NF YA and PRMT5 in LNCaP cells, and found that treatment of cells with a pan PKC inhibitor GFX completely restored the expression of NF YA and PRMT5 at mRNA and protein level (Fig. 5C–E). The observed increase in NF YB mRNA in cells treated with PMA plus GFX was likely due to the effect of GFX alone, because GFX treatment only increased NF YB expression at the mRNA level but had no effect on the expression of PRMT5, NF YA, and NF YC (Supplementary Fig. S2). Consistent with a role for NF Y in regulating PRMT5 transcription via the NF Y binding sites in the proximal promoter region, PMA treatment resulted in almost

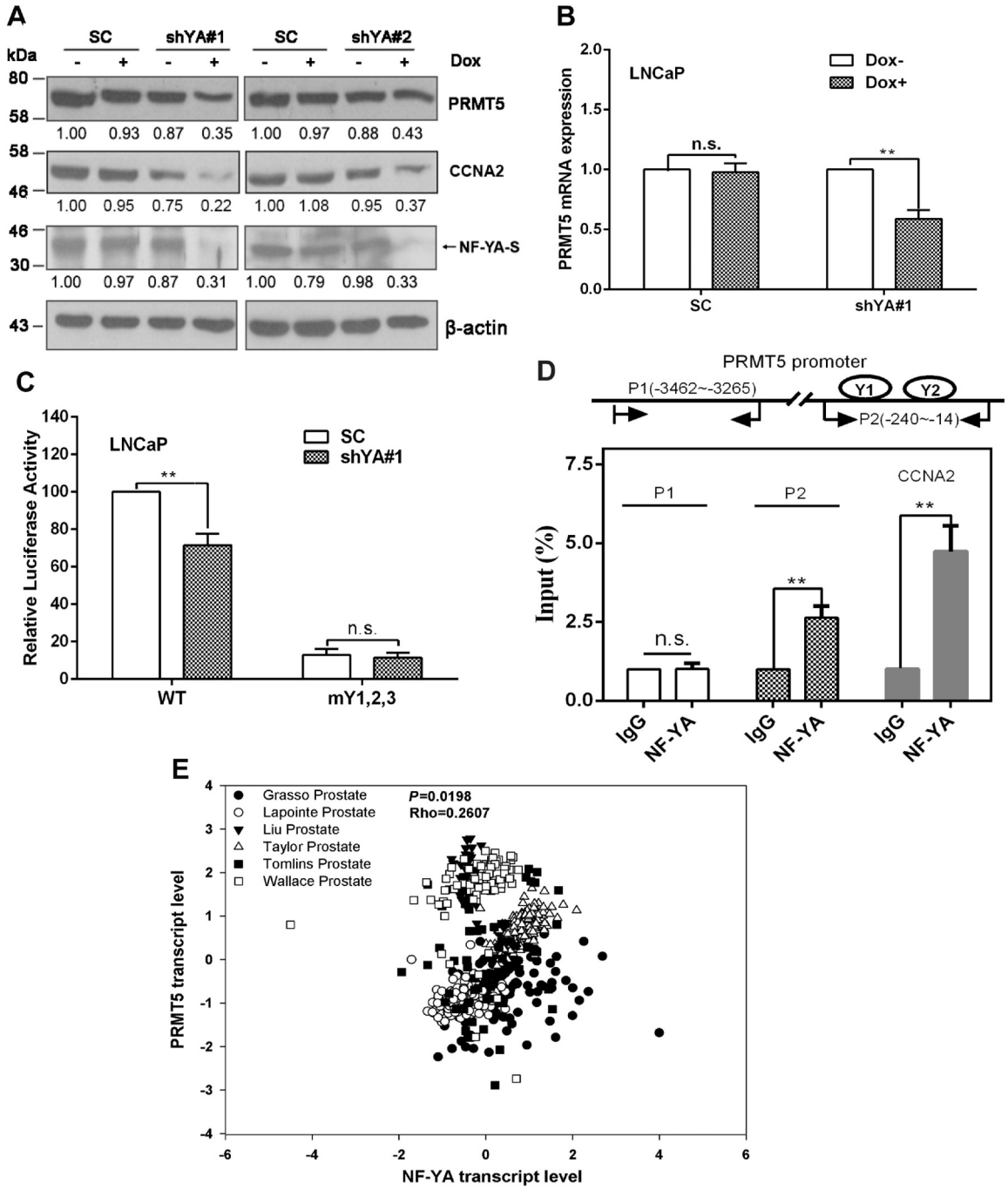

Fig. 3. NF-Y is essential for PRMT5 expression in LNCaP cells. (A) NF-YA knockdown inhibits PRMT5 expression. Doxycycline (Dox) was added at 1 μg/ml for 96 h to induce NF-YA knockdown in ShYA#1 and shYA#2 stable cell lines, and total cell lysate was used for immunoblotting analysis of PRMT5, shorter isoform of NF-YA (NF-YA-S), CCNA2 and β-actin. Shown are representative blots from three independent experiments, and the numbers indicate relative fold changes analyzed by Image J. (B) Knockdown of NF-YA inhibits PRMT5 mRNA expression. shRNA expression was induced by Dox for 72 h, and qRT-PCR was performed to determine the mRNA level of PRMT5. Results are mean ± SEM from four independent experiments, and Student's *t* test was used for statistical analysis (**$p < 0.01$). (C) Knockdown of NF-YA decreases the PRMT5 proximal promoter activity. One microgram of plasmids encoding SC or shYA1# (with Dox induction) was co-transfected with 0.5 μg of the B5 reporter gene plasmid (WT) or the mutant reporter gene (mY1,2,3), along with 100 ng of pRL-TK into LNCaP for 48 h, and dual-luciferase reporter assays were performed and analyzed. Luciferase activities are presented as percentage from at least three independent experiments. **$p < 0.01$. (D) NF-YA binds to the two inverted CCAAT boxes. Shown (top) is a schematic of the two regions (P1 and P2) in the PRMT5 promoter for ChIP analysis. Results (bottom) are mean ± SEM from four independent experiments (**$p < 0.01$). The binding of NF-YA to the CCNA2 promoter was used as a positive control. (E) The transcript level of NF-YA positively correlates with the transcript level of PRMT5 in prostate cancer. Data shown are a meta-analysis from six independent studies deposited in Oncomine database.

75% reduction of the NF YA binding to the proximal promoter region of PRMT5 (Fig. 5F). In agreement with previous findings that PMA inhibits cell growth and induces apoptosis only in LNCaP, but not in DU 145 and PC 3 cells [25,26], PMA treatment did not cause any significant change in NF YA and PRMT5 expression in PC 3 cells (Fig. 5G). Additionally, PMA did not have any effect on NF YA and PRMT5 expression in A549 cells (Fig. 5H). Thus, PMA treatment appears to have a specific effect on the expression of NF YA and PRMT5 in LNCaP cells.

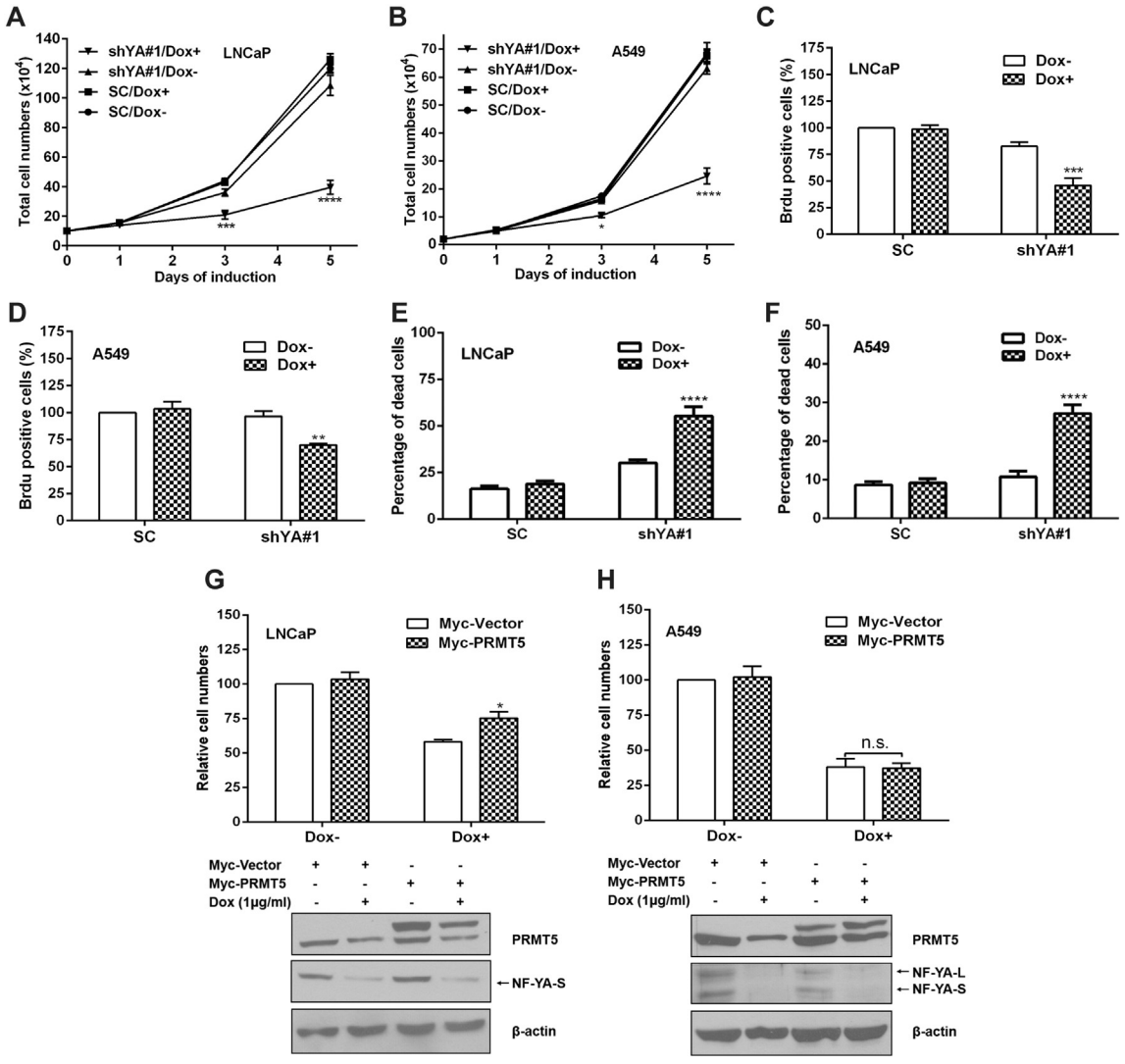

Fig. 4. NF-Y regulation of PRMT5 expression is required for prostate cancer cell growth. (A and B) Knockdown of NF-YA inhibits cell growth in LNCaP (A) and A549 (B). Stable cell lines SC and shYA#1 were induced with 1 μg/ml of doxycycline (Dox+) to express shRNAs or without treatment (Dox−) for the indicated times, and cell numbers were counted using hemocytometer. Results from four independent experiments in duplicate are presented as mean ± SEM. Statistical significance (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$) was determined by two-way ANOVA followed by Tukey's test. (C and D) Knockdown of NF-YA decreases BrdU-incorporated positive cells in LNCaP and A549 cells. SC and shYA#1 stable cell lines were induced with 1 μg/ml of Dox (Dox+) or without treatment (Dox−) for 84 h, followed by BrdU treatment for another 8 h. Number of BrdU-positive cells was determined using Image J software (total cell number > 1000, n = 10). Results obtained from four independent experiments in duplicate are presented as mean ± SEM. Statistical significance (**$p < 0.01$; ***$p < 0.001$) when compared with SC was determined by two-way ANOVA followed by Dunnett's test. (E and F) Effect of NF-YA knockdown on cell death. Stable and inducible cell lines targeting NF-YA (shYA#1) or the SC control were cultured in 6 cm dishes, and induced with 1 μg/ml of Dox (Dox+) or without treatment (Dox−) for 72 h. Cells were trypsinized and counted to determine the percentage of dead cells by Trypan blue exclusion method. (G and H) Overexpression of PRMT5 rescues cell growth inhibition induced by NF-YA knockdown in LNCaP cells, but not in A549. LNCaP and A549 stable cell lines expressing shYA#1 were induced with 1 μg/ml of Dox (Dox+) or without induction (Dox−) for 48 h, followed by transient transfection with pCMV-Myc (Myc-vector) or pCMV-Myc-PRMT5 (Myc-PRMT5) and incubation for another 48 h. Top, results are presented as mean ± SEM from three independent experiments. Statistical significance was determined by two-way ANOVA followed by Tukey's test. *$p < 0.05$; n.s., no significance. Bottom, the expression level of PRMT5 and NF-YA was determined by immunoblotting analysis. Shown are representative blots from three independent experiments. Note that the expression of both NF-YA longer isoform (NF-YA-L) and shorter isoform (NF-YA-S) was detectable in A549 cells whereas the expression of NF-YA-S was detectable in LNCaP cells only.

3.6. c Fos mediates the PKC signaling to regulate PRMT5 transcription via down regulation of NF YA expression

As AP 1 proteins c Fos and c Jun are downstream transcription factors of PKC that can be induced by PMA [30 32], we confirmed that PMA treatment indeed induced expression of c Fos and c Jun in LNCaP cells (Fig. 6A). However, overexpression of c Fos, but not c Jun, inhibited the PRMT5 reporter gene activity (Fig. 6B). Consistent with its effect on the PRMT5 reporter gene activity, overexpressed c Fos, but not c Jun, decreased PRMT5 mRNA (Fig. 6C) and protein expression (Fig. 6D). We found that NF YA expression at both mRNA and protein levels was also inhibited by c Fos (Fig. 6C and D). These results suggest that c Fos may mediate the PKC signaling to down regulate the expression of NF YA and PRMT5. To test this, we generated a shRNA construct targeting c Fos and observed that knockdown of c Fos increased the PRMT5 reporter gene activity by 54% (Fig. 6E). Further, we used the shRNA construct to establish an inducible stable cell line to knock down c Fos, and observed that PMA induced NF YA and PRMT5 down regulation was partially restored when c Fos was knocked down (Fig. 6F and G). Since the ENCODE ChIP seq data from the UCSC database (http://genome.ucsc.edu/ENCODE/) show that c Fos also binds to the proximal promoter region in HeLa S3 and K562 cells, we were interesting to know whether c Fos has any direct impact on the PRMT5 promoter activity in LNCaP cells. To this end, we examined the effect of c Fos overexpression or knockdown on the WT and the mutant PRMT5 reporter gene activity. As shown in Fig. 6H and I, we found that

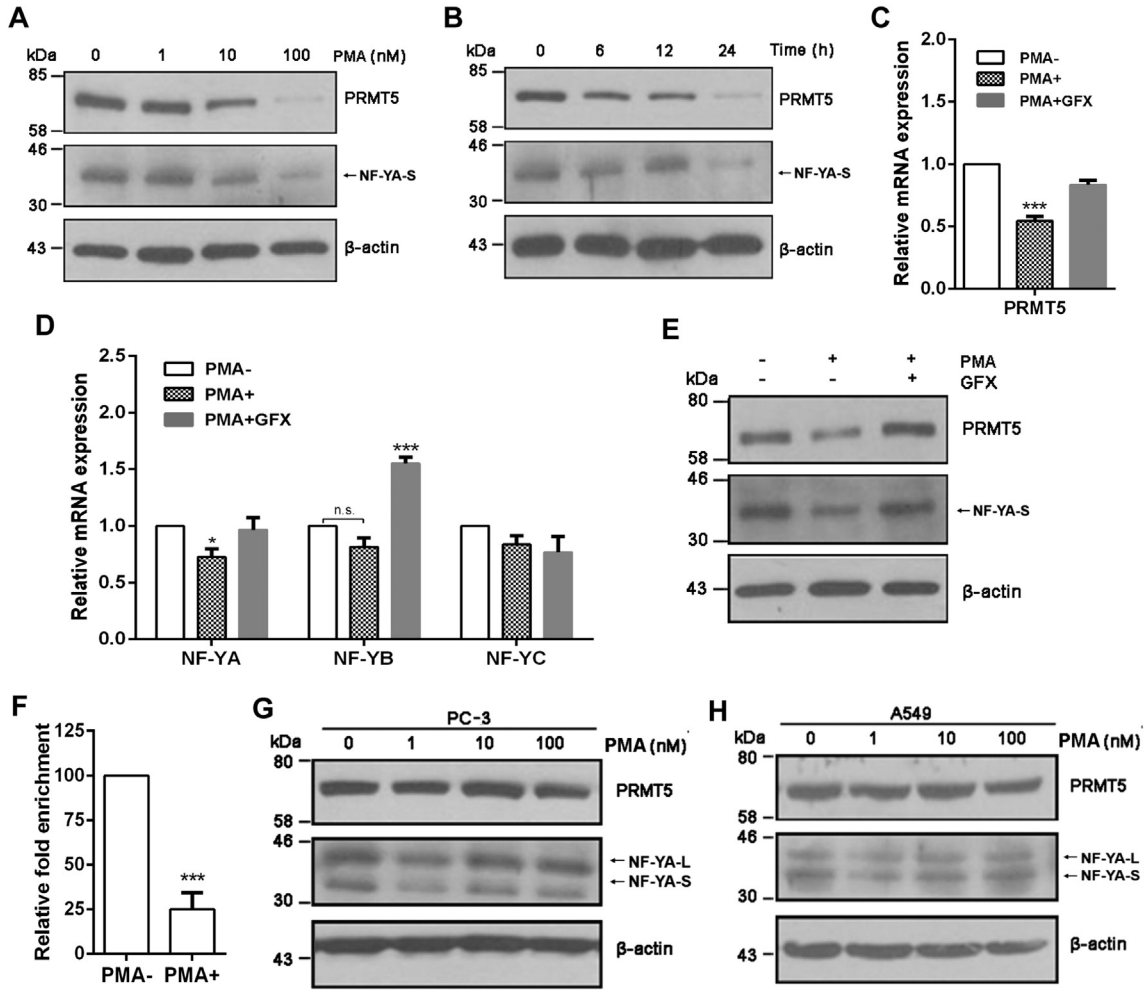

Fig. 5. PKC negatively regulates PRMT5 expression in LNCaP. (A and B) The PKC activator PMA inhibits NF-YA and PRMT5 expression in a dose- and time-dependent manner. LNCaP cells were treated with PMA at the indicated doses (A) for 24 h or treated with 100 nM of PMA for the indicated time points (B), and total cell lysate was used for immunoblotting analysis of PRMT5 and NF-YA expression. (C and D) A pan-PKC inhibitor inhibits PMA-induced down-regulation of PRMT5 and NF-YA at the mRNA level. LNCaP cells were treated with 100 nM of PMA in the presence or absence of a pan-PKC inhibitor GFX (200 nM) for 24 h, and relative mRNA level of PRMT5 (C) or NF-YA, NF-YB and NF-YC (D) was determined by qRT-PCR. Results from three independent experiments are presented as mean ± SEM in C and D, and statistical significance ($^{*}p < 0.05$, $^{***}p < 0.001$) was determined by one-way ANOVA followed by Tukey's test. (E) PKC inhibition restores NF-YA and PRMT5 expression at the protein level in cells treated with PMA. LNCaP cells were treated with 100 nM of PMA in the presence or absence of GFX (200 nM) for 24 h, then NF-YA and PRMT5 expression was analyzed by immunoblotting. Representative blots from three independent experiments are shown. (F) PMA treatment decreases NF-YA binding to the PRMT5 promoter. ChIP analysis was conducted using anti-NF-YA antibody to determine the binding of NF-YA to the two CCAAT boxes in the proximal promoter region of PRMT5. $^{***}p < 0.001$ (Student's *t* test). (G and H) PMA does not significantly affect the expression of NF-YA and PRMT5 in PC-3 and A549. PC-3 and A549 cells were treated with PMA at the indicated concentration for 24 h, and total cell lysate was used for immunoblotting detection of NF-YA and PRMT5 expression. PMA , DMSO treatment (Fig. 5C–F).

overexpression of c Fos decreased the WT PRMT5 reporter gene activity by 62.3%, but had no effect on the mutant reporter gene activity in which all three NF Y binding sites were mutated (mY1,2,3). In contrast, transient knockdown of c Fos remarkably increased the WT PRMT5 reporter gene activity, but had no effect on the mutant reporter gene activity. These results provide evidence that c Fos indeed mediates, at least partially, the PKC signaling to negatively regulate PRMT5 transcription via down regulation of NF YA in LNCaP cells.

4. Discussion

It has been reported that PRMT5 may function as an oncogene to promote cancer cell growth [1 3,5 7,9,10]. Although NF Y directly regulates transcription of many target genes to control cell cycle progression, cell proliferation and cell survival [12,13,15,17], our finding that NF Y transcriptionally activates PRMT5 expression suggests that NF Y may also regulate cancer cell growth by controlling the expression level of PRMT5, an emerging epigenetic enzyme that functions as an oncogene in human cancers [1]. For example, E2F1 is a member of the E2F family transcription factor required for transactivation of target genes involved in cell cycle progression in cancer cells [56]. Because the transcriptional activity of E2F1 is under the control of the tumor suppressor Rb, loss of Rb leads to constitutive activation of E2F1 and cancer development [57]. Interestingly, PRMT5 can epigenetically silence transcription of Rb [9]. Thus, activation or overexpression of NF Y may lead to PRMT5 overexpression, by which Rb is silenced and E2F1 is activated, providing another pathway to promote cell cycle progression in cancer cells that harbor the wild type Rb gene [9]. As NF Y also regulates the transcription of the same target genes such as E2F1 [58], future studies of how NF Y coordinates the regulation of PRMT5 expression and other target genes will likely provide novel insights into the oncogenic role of both NF Y and PRMT5 in cancer cells.

Recent evidence indicates that PRMT5 is overexpressed in multiple human cancers [3 11], though it is unknown how PRMT5 expression is regulated by cancer signaling. In leukemia and lymphoma cells, down regulation of several miRNAs contributes to PRMT5 overexpression [9,10]. We have provided several lines of evidence that NF Y regulates PRMT5 transcription via the binding to the two CCAAT boxes in the proximal promoter region of PRMT5. First, mutagenesis analyses showed that mutation of the two CCAAT boxes in the proximal

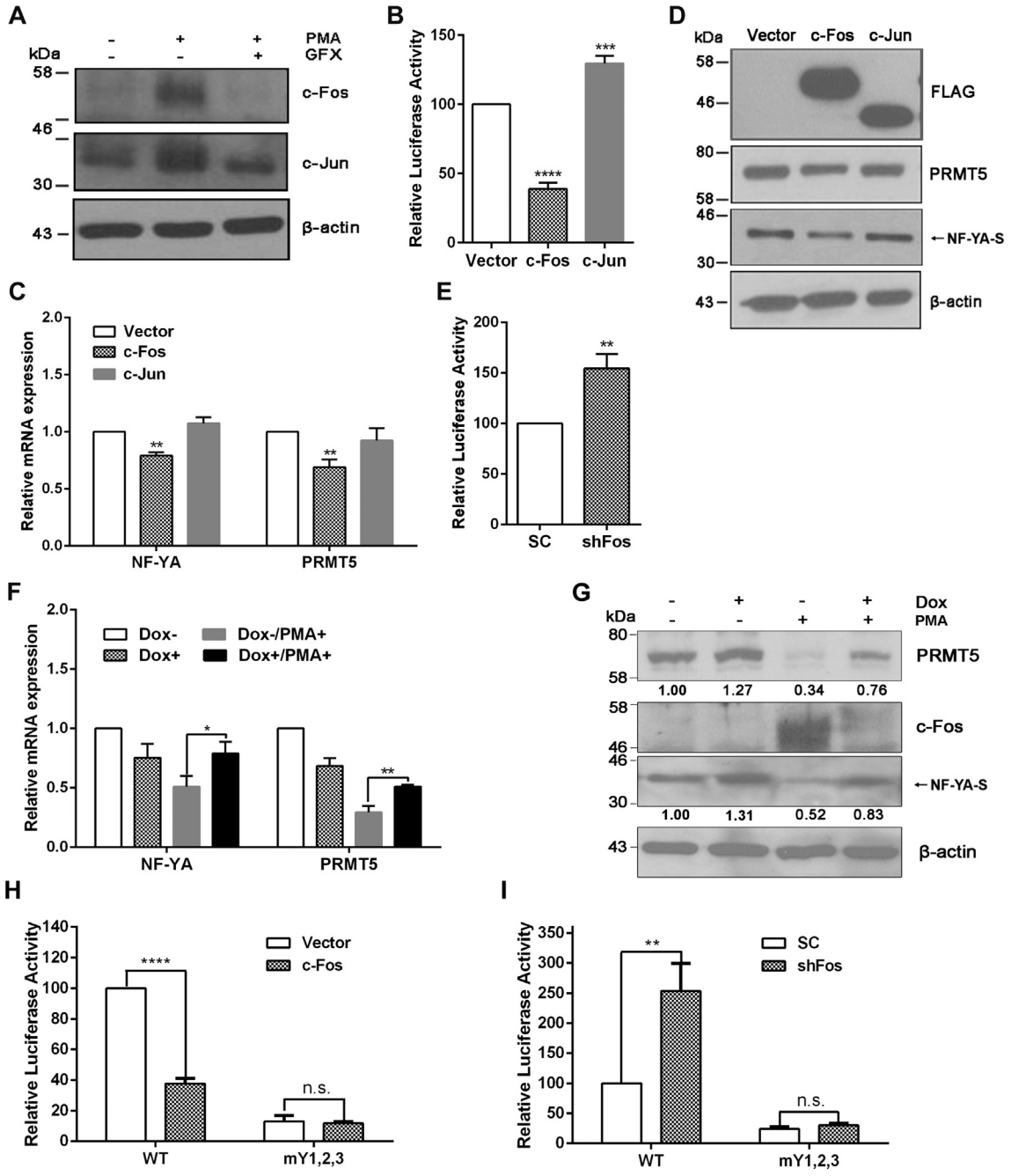

Fig. 6. c-Fos mediates the PKC signaling to down-regulate PRMT5 expression via NF-YA. (A) PMA increases c-Jun and c-Fos expression in LNCaP. LNCaP cells were treated with 100 nM of PMA in the presence or absence of GFX (200 nM) for 24 h, and the expression of c-Fos and c-Jun was determined by immunoblotting. (B) Overexpression of c-Fos, but not c-Jun, inhibits the PRMT5 promoter activity. One microgram of pCMV-FLAG (Vector), pFLAG-c-Fos (c-Fos) or pFLAG-c-Jun (c-Jun) was co-transfected with 0.5 μg of the wild-type (B5) reporter gene, along with 0.1 μg of pRL-TK into LNCaP cells. The luciferase activity was determined 24 h after the transfection. Results from six independent experiments in triplicate are presented as mean ± SEM, and statistical significance (***$p < 0.001$, ****$p < 0.0001$) was determined using one-way ANOVA followed by Dunnett's test. (C and D) Overexpression of c-Fos, but not c-Jun, inhibits NF-YA and PRMT5 expression. LNCaP cells were transfected with 3 μg of the indicated plasmids as described in B. The mRNA and protein expression of NF-YA and PRMT5 was determined by qRT-PCR (C) and immunobloting (D), respectively. Results from at least three independent experiments are presented as mean ± SEM. Statistical significance (**$p < 0.01$) was determined by using one-way ANOVA followed by Dunnett's test. (E) Knockdown of c-Fos increases the PRMT5 promoter activity. The SC or c-Fos shRNA (shFos) was co-transfected with 0.5 μg of the wild-type (B5) reporter gene, along with 0.1 μg of pRL-TK into LNCaP cells. The luciferase activity was determined 48 h after the transfection. **$p < 0.01$ versus SC (Student's t test). (F and G) Knockdown of c-Fos partially rescues NF-YA and PRMT5 expression. Stable cell line that can inducibly express a c-Fos shRNA was induced with 1 μg/ml of doxycycline (Dox +) or without treatment (Dox) for 48 h. Cells then were treated with 100 nM of PMA (PMA +) or DMSO (PMA) for another 24 h, followed by determination of the mRNA expression (F) and protein expression (G) of NF-YA and PRMT5. Statistical significance (*$p < 0.05$, **$p < 0.01$) was determined by two-way ANOVA followed by Tukey's test. The numbers in G indicate the relative expression level of each protein analyzed by Image J software. (H and I) c-Fos decreases PRMT5 promoter activity mainly through CCAAT boxes. The indicated plasmids were transfected into LNCaP cells, and the luciferase assays were performed following the same procedure as described in B and E, respectively. Results from three independent experiments in triplicate are presented as mean ± SEM, and statistical significance (**$p < 0.01$, ****$p < 0.0001$).

promoter region resulted in 70% reduction in the luciferase reporter gene activity in three different cancer cell lines (Fig. 2C E). Second, endogenous NF YA also specifically bound to the proximal promoter region containing the two CCAAT boxes in LNCaP cells (Fig. 3D). Third, knockdown of NF YA not only inhibited the PRMT5 promoter driven luciferase report gene activity but also decreased the expression of PRMT5 at both mRNA and protein levels (Fig. 3A C). We also show that the PKC/c Fos signaling negatively regulates PRMT5 expression

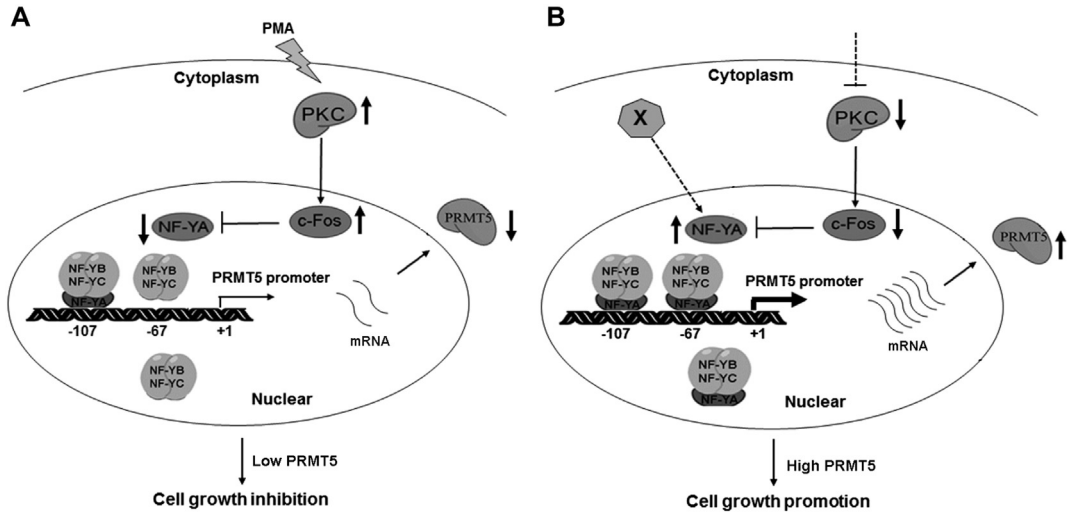

Fig. 7. Model for the regulation of PRMT5 expression by the PKC-c-Fos-NF-Y signaling in human cancer. (A) The PKC signaling negatively regulates PRMT5 expression in a c-Fos- and NF-Y-dependent manner in LNCaP cells. In response to PMA treatment, activation of PKC leads to the induction of c-Fos, which in turn suppresses NF-YA transcription and results in down-regulation of PRMT5. As a result, cell growth is inhibited. (B) Proposed mechanisms underlying up-regulation of PRMT5 expression in cancer cells. Two possible mechanisms may underlie PRMT5 overexpression in human cancers. One is the inactivation or down-regulation of PKC by cell signaling, and the other is direct activation or up-regulation of NF-YA by cell signaling that remains to be identified (X). Dashed lines indicate unknown factors that remain to be identified. Thick solid arrows illustrate the up-regulation or down-regulation of the indicated protein.

via down regulation of NF YA transcription in LNCaP prostate cancer cells (Figs. 5 7A). Although the mechanism by which c Fos represses NF YA transcription remains to be investigated, it is interesting to note that our preliminary analysis of the NF Y promoter identified three consensus AP 1 binding sites within the 6 kb promoter region. It is therefore possible that c Fos may directly repress NF YA transcription by binding to these consensus AP 1 binding sites. Alternatively, c Fos may indirectly repress NF YA transcription through a secondary effect (e.g., up regulation of a transcriptional repressor of NF YA). Neverthe less, our findings suggest that cell signaling may up regulate PRMT5 ex pression by down regulation of PKC or by direct up regulation of NF YA to promote cancer cell growth (Fig. 7B). This is further supported by the fact that several isozymes of PKC are down regulated in human cancers [59]. Indeed, a preliminary analysis of the Oncomine database shows that the transcript level of several PKC isozymes inversely correlates with the transcript level of PRMT5 in prostate cancer and lung cancer (Supplementary Fig. S3). It will be interesting to see whether down regulation of these PKC isozymes correlates with PRMT5 overexpression at the protein level in human cancer tissues.

The cell growth promoting role of PRMT5 is mediated by controlling the expression of target genes or by post translational modification of signaling molecules that are involved in cell cycle progression, apoptosis and DNA repair [1]. Although knockdown of PRMT5 in LNCaP cells in hibits cell proliferation [60], the downstream signaling mediating this effect remains unknown. A previous study suggests that PRMT5 may be required for the transcriptional activity of AR in a luciferase reporter gene assay [61]. Given that PMA induced down regulation of PRMT5 is mainly observed in AR positive LNCaP cells, but not in AR negative DU 145 and PC 3 cells, it is plausible to hypothesize that down regulation of PRMT5 by PMA in LNCaP cells may contribute to the suppression of LNCaP cell growth and induction of apoptosis by attenuating the AR activity [61]. As a recent report shows that PMA treatment in LNCaP cells can down regulate AR expression [62], it would be interesting to examine whether PRMT5 has any effect on AR expression. Alternatively, PMA induced PRMT5 down regulation may contribute to PMA induced apoptosis by enhancing the activity of p38δ, a major serine/threonine protein kinase mediating PMA induced apoptosis in LNCaP cells [26]. Support for this notion comes from a recent observation that PRMT5 forms a complex with p38δ and suppresses PKCδ and p38δ dependent signaling in keratinocytes [63]. Future studies to distinguish these possibilities will provide a novel insight into the regulatory role of PRMT5 in prostate cancer cells.

In summary, we have identified NF Y as the major transcriptional activator of PRMT5 in multiple cancer cell lines, and demonstrated that the PKC/c Fos signaling negatively regulates PRMT5 expression in LNCaP prostate cancer cells through down regulation of NF YA tran scription. Because down regulation of several PKC isozymes correlates with human cancer development and progression [59], further analysis of the interplay between PRMT5 and the PKC/c Fos signaling in human cancer will provide novel insights into the oncogenic role of RPMT5 in human cancers.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2014.09.015.

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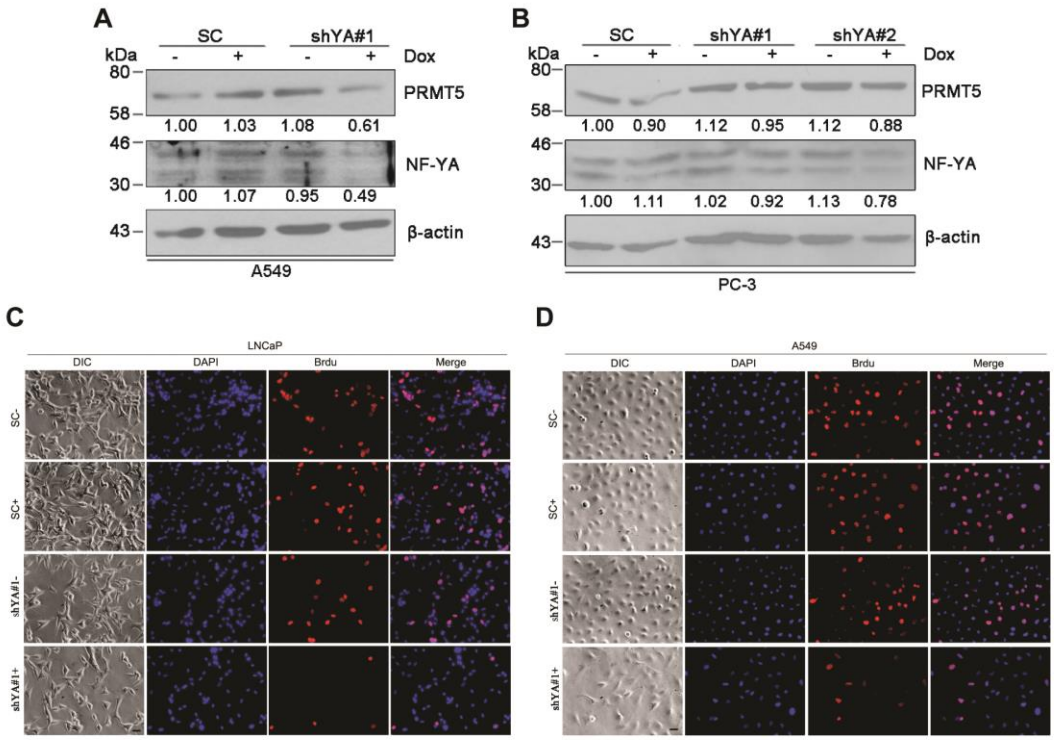

Fig. S1. Effect of PRMT5 knockdown on cell proliferation in LNCaP and A549 cells. (A) Knockdown of NF-YA decreases PRMT5 expression in A549 cells. A549 stable cell lines expressing shYA#1 or the scrambled control (SC) were induced to knock down NF-YA by 1 μg/ml of doxycycline (Dox) for 96 hours. Immunoblotting was applied to analyze expression of NF-YA and PRMT5. The number of values indicates the relative expression determined by Image J. (B) PC-3 stable cell lines expressing shYA#1 or shYA#2 or the scrambled control (SC) were induced to knock down NF-YA by 1 μg/ml of doxycycline (Dox) for 96 hours. Results were analyzed as in (A). (C and D) Knockdown of NF-YA inhibits Brdu incorporation in LNCaP and A549 cells. LNCaP and A549 stable cell lines were induced with and without Dox (1 μg/ml) for 84 hours, followed by BrdU treatment for another 8 hours. Cells were fixed and immunostained with a BrdU-specific antibody (Red). The nucleus was stained with DAPI (Blue). Scale bar: 50 μm.

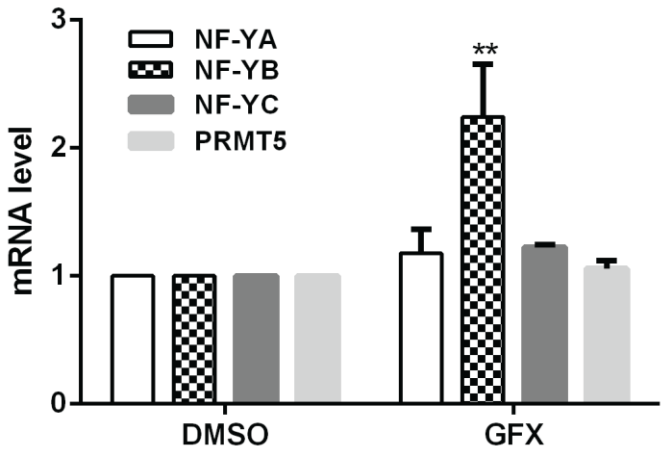

Fig. S2. Effect of GFX on mRNA expression of PRMT5 and NF-Y subunits. LNCaP cells were treated with GFX (200 nM) or DMSO for 24 hours. The mRNA expression of NF-YA, NF-YB, NF-YC and PRMT5 was determined by qPCR. Student's *t* test was used for statistical analysis (**, $p<0.01$).

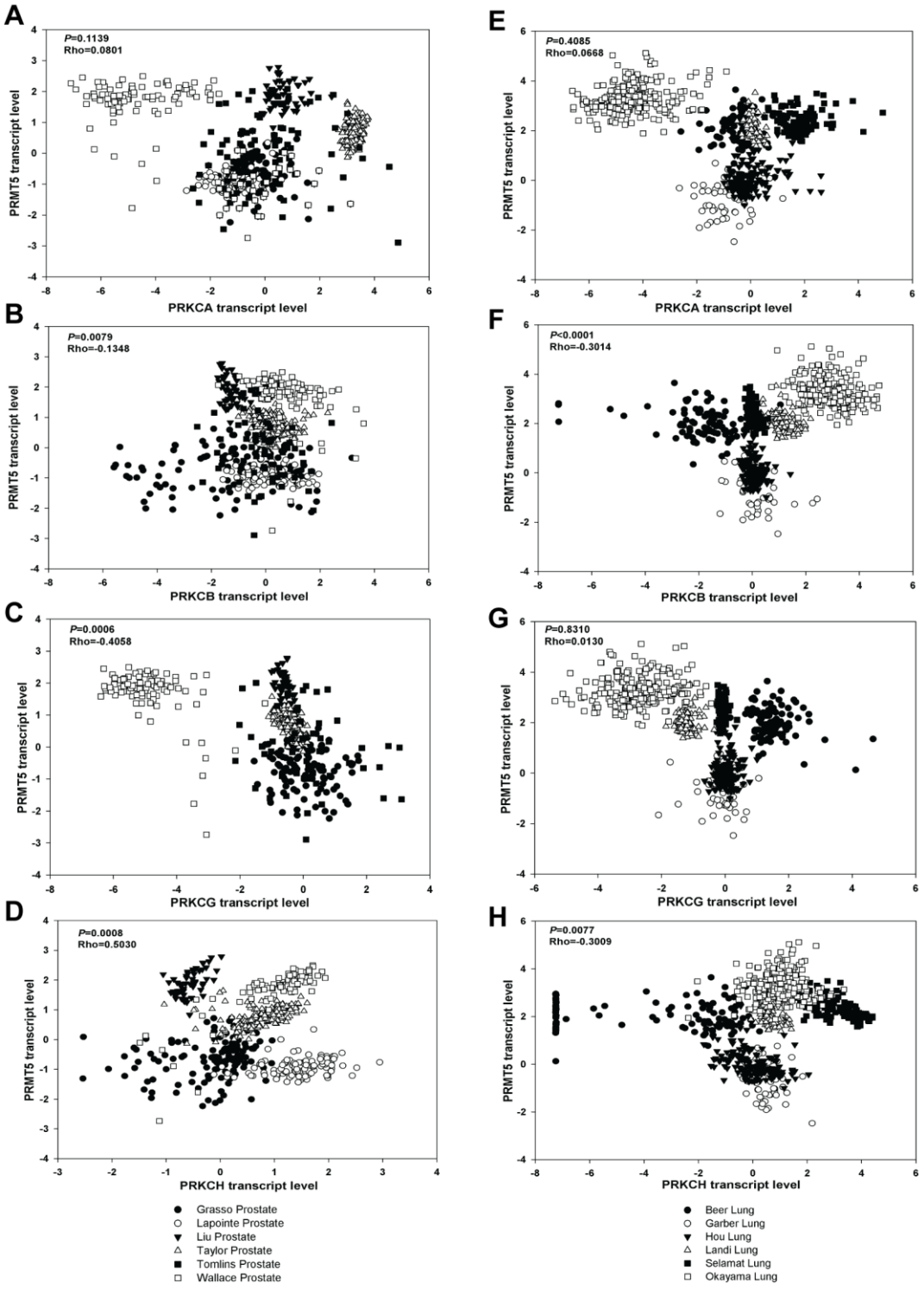

Fig. S3. The correlation between PKC isozymes and PRMT5 transcript in cancer tissues. Expression of several PKC isozymes correlates with expression of PRMT5 in prostate cancer (A-D) and lung cancer (E-H). Data shown are from six independent studies (each study has more than 60 samples) deposited in Oncomine database (www.oncomine.org). All these studies were pooled for correlation analysis, and a random-effects model was employed for the meta-analysis of each pair.